

AFAMRL-TR-80-125

AFAMRL-TR-  
80-125  
ADA102259  
citation



**PROCEEDINGS OF THE ELEVENTH CONFERENCE  
ON ENVIRONMENTAL TOXICOLOGY  
18, 19, AND 20 NOVEMBER 1980**

UNIVERSITY OF CALIFORNIA, IRVINE  
OVERLOOK BRANCH, P. O. BOX 3067  
DAYTON, OHIO 45431

JUNE 1981

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## TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals, "Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER



ANTHONY A. THOMAS, MD  
Director  
Toxic Hazards Division  
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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER AFAMRL-TR-80-125	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) PROCEEDINGS OF THE ELEVENTH CONFERENCE ON ENVIRONMENTAL TOXICOLOGY 18, 19, and 20 NOVEMBER 1980		5. TYPE OF REPORT & PERIOD COVERED CONFERENCE PROCEEDINGS
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s)		8. CONTRACT OR GRANT NUMBER(s) F33615-80-C-0512
9. PERFORMING ORGANIZATION NAME AND ADDRESS University of California, Irvine P.O. Box 3067, Overlook Branch Dayton, Ohio 45431		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62202F, 6302, 01, 15
11. CONTROLLING OFFICE NAME AND ADDRESS Air Force Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio 45433		12. REPORT DATE June 1981
		13. NUMBER OF PAGES 411
		15. SECURITY CLASS. (of this report) UNCLASSIFIED
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report)  Approved for public release; distribution unlimited.		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Behavioral Toxicology      Hydrocarbon Fuels      Genetic Toxicology Pulmonary Physiology      Short-Term Exposure Limits      Carcinogenesis Emphysema      Liquid Gun Propellants Oxidant Gases      Epidemiology <u>Sensitization</u> <u>Solvents</u>		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Subjects of presentations and discussions included behavioral consequences of exposure to occupational environments, development of emphysema after exposure to chemicals, epidemiology of industrial solvents and genetic factors in susceptibility to environmental agents. The toxic effects of specific fuels and chemicals used by the Armed Forces were also discussed.		

## PREFACE

The Eleventh Conference on Environmental Toxicology was held in Dayton, Ohio on 18, 19, and 20 November 1980. Sponsor was the University of California, Irvine under the terms of Contract F33615-80-C-0512 with the Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio. Arrangements were made by the Toxic Hazards Research Unit of the University of California, Irvine. Donald L. Dungworth, Ph.D., Professor and Chairman, Department of Pathology, School of Veterinary Medicine, University of California, Davis served as Conference Chairman, and Mrs. Lois Doncaster, University of California, Irvine served as Conference Coordinator. Acknowledgement is made to Ms. Mildred Pinkerton and Ms. Joyce Sizemore for their significant contributions and assistance in the preparation of this report.



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## INTRODUCTORY ADDRESS

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Good morning, ladies and gentlemen, I'm Col. George Mohr, a Flight Surgeon, currently assigned at Wright-Patterson as the Commander of the Aerospace Medical Research Laboratory. It's my great pleasure to welcome you here on this first visible day of winter although the calendar doesn't quite agree. I'm happy to see that you all made it safely; I presume that most of you spent the evening here because I understand the highways in the Dayton area are still hazardous this morning. However, I'm sure the warmth of fellowship and the quality of the science that will engage your minds over the next couple of days will make up for the inclement weather.

I have two tasks this morning. The first of these is to make a few introductory remarks; perhaps to throw out a challenge or two and finally to introduce one of our most distinguished guests who will make the formal remarks as to the conduct of the sessions.

First of all, while I'm not a toxicologist, I am a Flight Surgeon and I share a common interest with most of you. Over the years some of us working in this area may have had an identity crisis. I've never in my own mind completely sorted out what a toxicologist is. Because when I look at my own laboratory, I see Veterinary Pathologists, Physiologists, Biochemists and Pharmacologists; in fact, I see a whole range of disciplines that somehow or other have learned to speak the same language and share a central interest in the health of our folks in the workplace as well as in maintaining a healthful environment for our friends in the surrounding community. It is apparent that over the last forty or fifty years, we have gone through a maturation process: learning to work together while educating a highly skilled category of scientists that are continuing to specialize. This is very important because a significant threat that can impact the well-being of the human race requires us to deal with a rapidly growing overburden of new chemical species entering our environment each year, each carrying the potential threat of degrading the quality of our life and unfortunately, in some instances, even shortening life. It's overwhelming to remember that just a few years ago when I was at the Headquarters Aerospace Medical Division, my colleagues told me there were over 30,000 important chemical materials that were needed to manufacture the tools and systems that make the Air Force an effective military force. Over 30,000 important chemicals. At least 10% of these were considered to be potentially dangerous. Some of them dangerous at relatively low levels; particularly if you speculated about the long-term effects of repeated and cumulative exposure during a working lifetime. Some of the early efforts my laboratory has taken to deal with this problem have demonstrated just how difficult it will be to finally resolve all these questions if we limit our activities to whole body studies using a variety of animal species, attempting through brute force to identify the dose-response relationships and extrapolate that information to man. The

rate at which new chemical species are being produced and the rate at which our understanding generates new questions (because as is always the case, as you begin to answer a question you learn how to ask a better question) may never permit us to catch up unless perhaps the whole nation decides to become toxicologists. Therefore, in the last three or four years, in my laboratory at least, we've begun to emphasize a new approach to the problem. There is no question for a chemical species that has a high risk based on good evidence, that we need to determine a dose-response relationship from in vivo animal studies, to set a credible human safety standard. But before we get to that stage, by understanding the pathways through which a chemical agent enters the body, whether by inhalation, ingestion, or absorption through the skin, by understanding how the chemical species is transported within the body of the animal, and is stored, metabolized, and ultimately excreted, and by understanding the toxicodynamics of the particular cell system, sub-cellular system, or enzyme system where one or more of the products of a particular chemical system really acts, we should be able to devise sensitive screening tests (you're all familiar with many of those that are now available). We should be able with increasing validity to select those particular agents which are most likely to truly represent a health hazard in order to concentrate on those agents which are both most ubiquitous and carry the greatest risk of exposure for our people. This kind of approach has been described by many names. We speak of it in terms of toxicodynamics, toxicokinetics, metabolic mechanisms, screening techniques and scaling laws, but it all involves a very complex wedding of the many skills that are represented in this room. It is therefore particularly gratifying for me to see a group like this come together each year. If my memory fails me not, I believe this is the 11th such annual gathering that has occurred under the sponsorship presently of the University of California, Irvine. I believe those of you from industry, academia, other government agencies and from the military departments have a very real opportunity here to enrich your own understanding, to infuse new ideas and, I believe, ultimately to develop practical solutions to the problem of ensuring human safety which is of great personal interest to me.



## SESSION I

### BEHAVIORAL CONSEQUENCES OF EXPOSURE TO OCCUPATIONAL ENVIRONMENTS

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ANIMAL MODELS OF TOXICITY: SOME COMPARATIVE DATA  
ON THE SENSITIVITY OF BEHAVIORAL TESTS

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INTRODUCTION

Although several federal agencies are charged with protecting the public from the adverse health effects of exposure to toxic substances, they differ in their concern over the sources of exposure, and the population at risk. Sources of exposure, for example, include the work place, the environment, the household, and food. A common concern of the federal regulatory agencies is the accurate prediction of the health consequences of exposure to toxicants through the development of animal models of toxicity.

The present paper is concerned with the development of animal models of behavior to serve as screens for neurotoxic compounds. Such an enterprise was in part the result of recent environmental legislation that specifically calls for the development of tests for behavioral disorders. The Clean Air Act of 1970, amended in 1977, was enacted to protect and enhance the quality of air resources so as to promote the public health and welfare. A pertinent section of this act reads as follows:

CLEAN AIR ACT (AMENDED 1977), Section 103 f(1)

"In carrying out research pursuant to this act, the Administrator shall give special emphasis to research on the short- and long-term effects of air pollutants on public health and welfare. In the furtherance of such research he shall conduct an accelerated research program --

(A) to improve knowledge of the contribution of air pollutants to the occurrence of adverse effects on health, including, but not limited to, BEHAVIORAL, physiological, toxicological, and biochemical effects;"

The Toxic Substances Control Act (1976) was enacted to regulate chemical substances and mixtures which present undue risk of injury to health or the environment. A pertinent section related to behavioral toxicology reads as follows:

TOXIC SUBSTANCES CONTROL ACT (1976). Section 4(b)(2)(A).

"The health and environmental effects for which standards for the development of test data may be prescribed include . . . BEHAVIORAL DISORDERS, cumulative or synergistic effects, and any other effects which may present an unreasonable risk of injury to health or the environment."

We shall focus the present discussion on a series of experiments designed to evaluate the utility of various behavioral tests as screens for neurotoxicity. In general, in order for a method to serve as a screen for toxicity it must conform to defined protocols, be sensitive, inexpensive, reproducible and interpretable and should exhibit a moderate specificity and broad application. The heterogeneous nature of behavior, however, together with the almost unparalleled complexity of the nervous system raises serious questions as to whether a single test will ever suffice as a primary screen for neurotoxicity. Because of this, a variety of tests were selected for study that included: (1) locomotor activity in a novel environment; (2) performance on a spatial memory task; (3) performance under schedules of positive reinforcement, and; (4) the development of a flavor aversion when toxicant exposure was previously paired with that flavor. These tests sample diverse aspects of behavior and, therefore, would be expected to have different sensitivities to toxicant exposure.

Our specific approach was to evaluate the relative sensitivity of these behavioral tests following acute exposure of rats to a variety of insecticides. Representative chemicals were selected from three classes of insecticide including the carbamates, the synthetic pyrethrins, and the formamidine insecticides.

Carbamate insecticides, short-acting reversible inhibitors of cholinesterases, are used to control pests commonly found on crops, in gardens, and in the household (Kuhr and Dorough, 1976). Two representative carbamates with diverse LD50's, Baygon and Carbaryl, were chosen to further assess the ability of various behavioral tests to detect effects of chemicals with similar structures but with different potencies. Synthetic pyrethrins represent a relatively new class of insecticide that have a purportedly low mammalian toxicity and do not act through inhibition of cholinesterases (Elliott et al., 1978; Elliott and Janes, 1978; Elliott, 1976; Miyamoto, 1976). Decamethrin was chosen since more toxicological information was available for it than for the other synthetic pyrethrins (e.g. Ray and Cremer, 1979). Formamidines also represent a relatively new class of insecticide that have been used successfully to control certain types of mites and other insects that have proven resistant to the carbamates and other cholinesterase-inhibiting toxicants (Hollingworth, 1976; Lund et al., 1979). Chlordimeform was selected on the basis of data available regarding its toxic effects in rodents (Matsumura and Beeman, 1976). These insecticides were selected for investigation because they represent bona fide neurotoxic substances; in a sense, then, this research may also be viewed as an attempt to validate the use of behavioral tests for the detection of neurotoxic potential.

Behavior is an integrated response of the organism, and therefore it is a truism that at some exposure level all chemicals will produce behavioral effects. If nothing else, death is the ultimate behavioral endpoint. In order for behavioral tests to be useful in the screening process, a practical requirement is that they be capable of detecting behavioral changes in the absence of overt signs of intoxication. Consequently, it is important to consider the dosage levels producing behavioral effects (i.e., ED50s) in the context of those required to produce overt toxicity (i.e., mortality or the LD50s).

## BEHAVIORAL TESTING

### FIGURE-EIGHT MAZE ACTIVITY

Motor activity was measured in a figure-eight maze as previously described (Reiter et al., 1975). Briefly, the maze consists of a series of interconnected alleys (10 x 10 cm) converging on a central open arena and covered with transparent acrylic plastic. Motor activity was detected by 8 phototransistor-photodiode pairs; one count was registered each time a light beam was interrupted. Eight mazes were housed in a sound-attenuated room that was maintained on a photoperiod identical to that of the animal quarters. In all experiments, adult CD male rats were individually tested for 2 hr. Independent groups of animals were used for each insecticide and each animal received only one exposure. Treatment groups were comprised of 9-12 animals.

### RADIAL ARM MAZE

Adult male Long-Evans-derived (hooded) rats were placed on a restricted water-availability regimen (15-30 min/day). Each rat was given one daily trial on the maze and seven to ten training trials were given before treatments were administered. The maze consisted of a center platform from which eight arms radiated, like spokes of a wheel, and at the ends of which a small quantity of water could be deposited (Olton and Samuelson, 1976; Eckerman et al., 1980). A trial consisted of placing the rat on the center platform and allowing it to move freely about the maze until (a) all eight arms had been traversed to the end and water was consumed from all locations, or (b) 5 min. had elapsed. The rat was then removed to its home cage where supplementary water was available as necessary to maintain deprivation. An observer recorded the order and time of each arm entrance. Rats were randomly assigned to treatment conditions and the observers were not told which rats received which treatments. Independent groups with 5-6 animals per treatment were used for each insecticide.

### SCHEDULE-CONTROLLED BEHAVIOR

Adult male Long-Evans-derived (hooded) rats were maintained by food deprivation at 350 g body weight and were trained to respond (lever press) under a fixed-ratio or a fixed-interval schedule of milk reinforcement. Under a fixed-ratio schedule, milk reinforcement was provided after completion of a fixed number of responses (either 10 or 40); this schedule generates fairly rapid rates of responding (e.g., 1-6 responses per second). Fixed-

interval schedules provide reinforcement for a response after a specified interval of time (180 sec) has elapsed since the previous reinforcement; this schedule generates much slower overall response rates (e.g., 0.1-0.5 responses per second) although local rates of responding generally increase as time within the interval elapses. Testing was carried out 5 days per week in isolation chambers during sessions that were 45-48 min. in duration. One group of eight rats performed under the fixed-interval 180-sec. schedule and was used to determine the effects of the carbamate insecticides. Another group of eight was used to determine the effects of chlordimeform; four animals performed under the fixed-ratio-10 schedule and four performed under the fixed-ratio-40 schedule. For the decamethrin determinations, another group of eight rats was trained to perform under a fixed-ratio-40 schedule. Once performances had stabilized, insecticides were administered in an irregular sequence no more than twice a week (once a week for decamethrin). Only treatment effects on overall rates of responding are considered in this presentation.

### FLAVOR AVERSION

Adult male, Long-Evans-derived (hooded) rats received daily access to tap water for 30 min. in test cages located in the colony room. When intakes had stabilized (typically in 7-10 days), all rats were given 30-min. access to a saccharin solution (0.1% w/v) and were then given the appropriate treatment approximately 20 min. after the saccharin availability. Control rats received either no treatment or the appropriate vehicle. Water was then made available for the next two daily sessions; on the third session rats were provided with both saccharin and tap water. Comparisons were made between treatment groups for saccharin preference expressed as the proportion of total fluid intake. For the chlordimeform study, rats initially received access to both saccharin solution and tap water. For the decamethrin study, all rats received corn oil via gastric intubation after water availability for a 5 day period preceding initial saccharin availability. Independent groups of animals were used for each insecticide and each animal received only one treatment. Treatment groups were comprised of 5-6 animals.

### CHEMICAL TESTING

#### CARBAMATE INSECTICIDES

Baygon (o-isopropoxyphenyl, N-methylcarbamate; Chemagro, Kansas City, MO; 97% pure) and Carbaryl (1-naphthyl, N-methylcarbamate; Union Carbide, South Charleston, W. V.; >99% pure) were dissolved in warm corn oil and administered i.p. in a volume of 0.5 ml/kg body weight. Injections were given 20 min. prior to testing except for the flavor-aversion experiment where it was given 20 min. after the initial saccharin availability and 3 days prior to the saccharin-aversion assessment. Dosages ranged from 0.228-8.0 mg/kg and 0.943-28.0 mg/kg for Baygon and Carbaryl, respectively.

## FORMAMIDINE INSECTICIDE

Chlordimeform HCl (N -(4-chloro-o-tolyl)-N, N-dimethylformamidine; NorAm Agriculture Products, Woodstock, IL; >99% pure) was dissolved in normal saline and administered i.p. in a volume of 1 ml/kg body weight. Doses (as salt) ranged from 1.25-40.0 mg/kg and were administered 10 or 20 min. prior to testing except in the flavor-aversion experiment where it was given 20 min. after initial saccharin availability and 3 days prior to the saccharin-aversion assessment.

## SYNTHETIC PYRETHROID INSECTICIDE

Decamethrin ( (S)- $\alpha$ -cyano-3-phenoxybenzyl (1R, 3R)-cis-3-(2,2-dibromovinyl)-2,2-dimethyl-cyclo-propanecarboxylate; Roussel Uclaf, Romanville, France; Technical Grade) was dissolved in warm corn oil and administered p.o. in a volume of 1 ml/kg body weight. Doses ranged from 1.0-16.0 mg/kg and were administered 120 min. prior to testing except in the flavor-aversion experiment where it was given 20 min. after initial saccharin availability and 3 days prior to saccharin-aversion assessment.

## RESULTS

### DOSE-RESPONSE DATA

Individual dose-response curves, grouped according to insecticide, are presented in Figures 1 - 4. Except for the results of the flavor-aversion test, data are presented as percent of either the noninjected or the vehicle-injected controls. For the flavor-aversion paradigm, data are presented as saccharin intake expressed as the percent of the total intake (i.e., saccharin plus tap water). With few exceptions, the dose-range employed in these studies encompassed the ED50 which is defined as the dose producing a 50-percent change in response.

### BAYGON

Doses of Baygon ranging from 2-8 mg/kg produced decreases in all behavioral measures except flavor aversion (Figure 1). For those behaviors which were affected, the ED50s range from 2.0-3.7 mg/kg. Baygon did not produce a flavor aversion; additional data not presented in Figure 1 indicated even a dose of 11 mg/kg produced no effect on this measure.

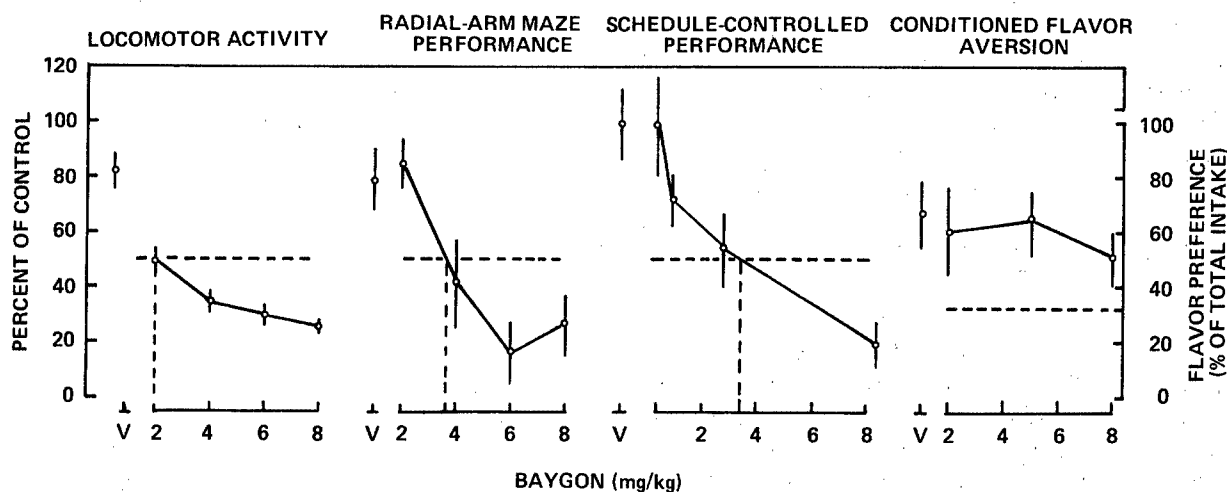


Figure 1. Behavioral effects of Baygon.

#### FIGURE LEGEND:

The left-most panel shows the effects of Baygon on locomotor activity measured in a figure-eight maze during a two-hour test session. Each symbol represents the average effect in 9-12 rats; vertical bars represent  $\pm 1$  SEM. The control value (100%) equals 376 counts. The next panel shows the effect of Baygon on the rate at which rats ( $N=5-6$ ) made selections on a radial-arm maze during a brief (5-min maximum) test session. Non-injected control (100%) equals 2.23 selections per minute. The third panel shows the effect of Baygon on the overall rate at which rats ( $N=8$ ) responded during 45-48 min test sessions under a FI 180-sec schedule of milk reinforcement. Non-injected control (100%) equals 0.24 responses per second. The right-most panel shows the effect of Baygon on the saccharin intake (expressed as a percentage of total intake) of rats ( $N=5-6$ ) in a flavor-aversion conditioning paradigm. With the exception of the flavor-aversion data, the dashed horizontal lines are drawn at 50% of control, and the dashed perpendicular lines indicate the dosage corresponding to the 50% value (i.e., the ED50). For the flavor-aversion data the horizontal line was drawn at one-half the value of the vehicle-control group.

#### CARBARYL

Dose-response curves for Carbaryl are shown in Figure 2. Doses ranging from 2.5-28 mg/kg produced decreases in all behavioral measures. For both motor activity and schedule-controlled behavior there appears to be a steeper dose-response function for Carbaryl than for Baygon which results in a greater overall decrease in response at the largest doses. Unlike Baygon, Carbaryl produced a flavor aversion but only at the largest dose. The ED50s for Carbaryl ranged from 5.5-21.1 mg/kg.

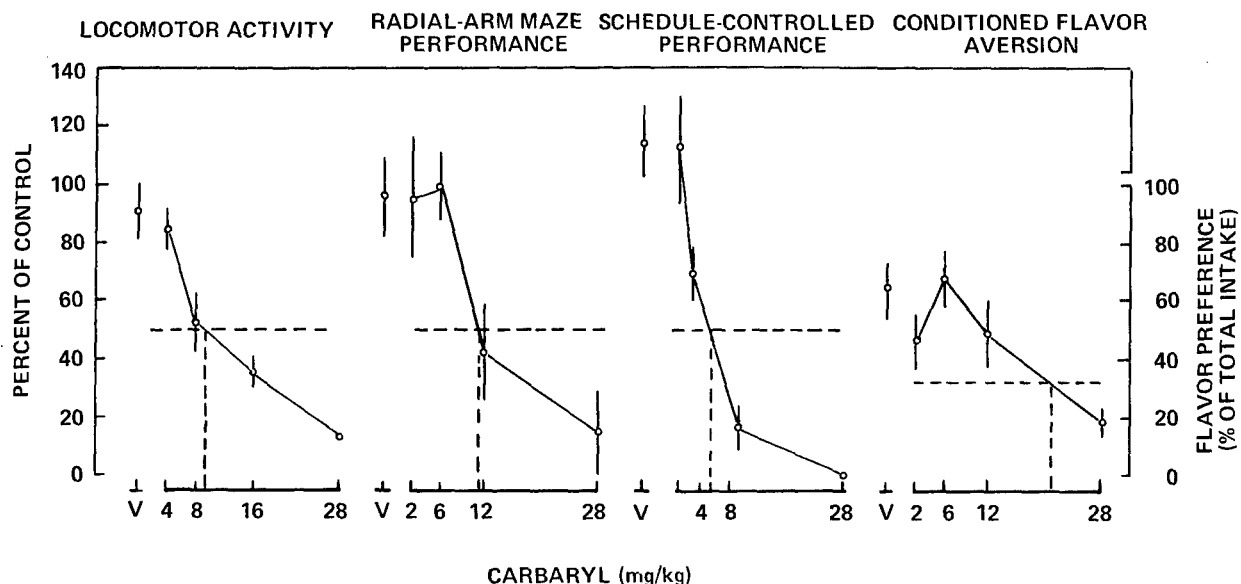


Figure 2. Behavioral effects of Carbaryl.

#### FIGURE LEGEND:

Control values (100%) equal 330 counts per two hours, 2.74 selections per minute, and 0.26 responses per second for locomotor activity, radial-arm-maze performance and schedule-controlled performance, respectively. See legend of Figure 1 for additional details.

#### CHLORDIMEFORM

Dose-response curves for chlordimeform are shown in Figure 3. Doses ranging from 2.5-40 mg/kg produced decreases in all behavioral measures. For schedule-controlled behavior, data are presented for performance under both an FR-10 (fixed ratio of 10 responses) and an FR-40 schedule of reinforcement. Surprisingly, the schedule with the lesser work requirement was associated with the smaller ED<sub>50</sub>. For locomotor activity, the smallest dose (5 mg/kg) produced a 50% decrease in response; higher doses were only slightly more effective in reducing activity. The ED<sub>50</sub>s ranged from 5-17.7 mg/kg.



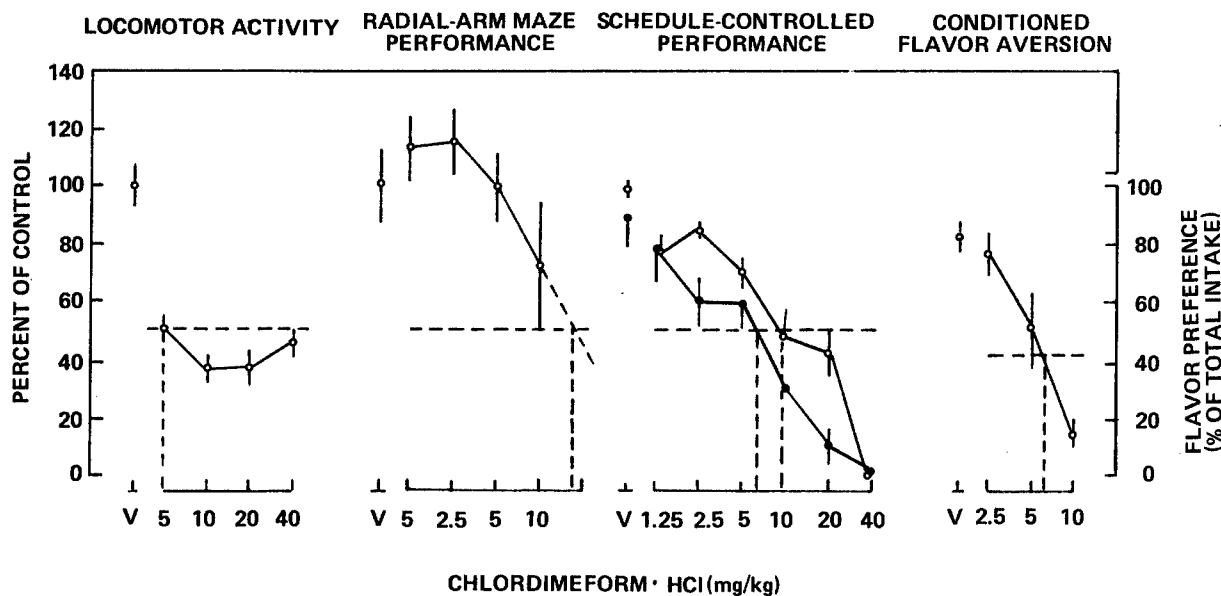


Figure 3. Behavioral effects of Chlordimeform.

#### FIGURE LEGEND:

Rats in the schedule-controlled experiment performed under an FR-10 (closed symbols, N=4) or an FR-40 (open symbols, N=4) schedule of reinforcement. Control values (100%) equal: 427 counts per two hours, 2.51 selections per minute, 0.94 responses per second (FR-10) and 2.25 responses per second (FR-40) for locomotor activity, radial-arm-maze performance and schedule-controlled performance, respectively. See legend of Figure 1 for additional details.

#### DECAMETHRIN

Doses of decamethrin ranging from 1-16 mg/kg produced decreases in all behavioral measures (Figure 4). This compound was the most potent of the insecticides in the flavor-aversion paradigm, with an estimated ED<sub>50</sub> of 1.6 mg/kg. Activity in the figure-eight maze was not further reduced when the dosage was increased from 8 to 16 mg/kg; a similar plateau in the dose-response curve can be seen in the flavor-aversion data. ED<sub>50</sub> values for decamethrin ranged from 1.6-7.6 mg/kg.

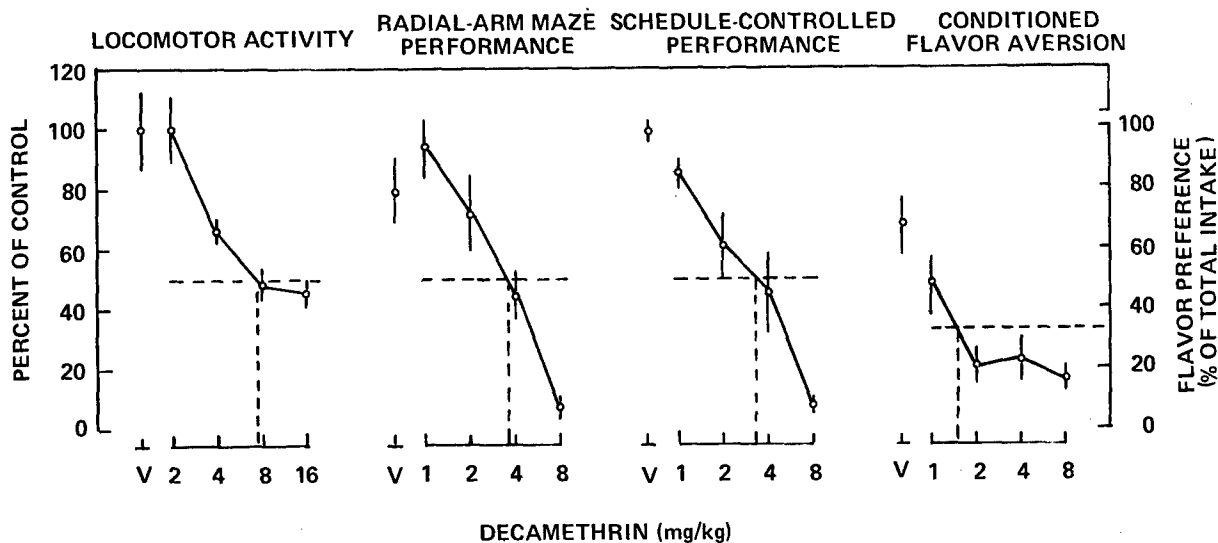


Figure 4. Behavioral effects of Decamethrin.

#### FIGURE LEGEND:

Rats (N=8) in the schedule-controlled experiment performed under an FR-40 schedule of reinforcement. Control values (100%) equal 401 counts per two hours, 3.31 selections per minute, and 3.22 responses per second for locomotor activity, radial-arm-maze performance and schedule-controlled performance, respectively. See legend of Figure 1 for additional details.

#### COMPARISONS OF ED50s

The ED50 values were estimated graphically from the data in Figures 1-4 and are presented for each behavioral test in Table 1. Considering the diversity of behavioral measures, there is remarkable agreement across tests. If one excludes the Baygon flavor-aversion data, for which the ED50 was indeterminate, the ratio of maximum to minimum ED50 ranged from a low of 1.7 to a high of 4.8. In general, figure-eight maze activity and schedule-controlled performance produced the lowest ED50s when these values were ranked across all insecticides.

#### BEHAVIORAL TOXICITY INDEX

Literature values for the LD50 of each insecticide, based on the appropriate route of administration, and the ED50s taken from Table 1 were used to calculate the Behavioral Toxicity Index (LD50/ED50). Several LD50 values were available for Carbaryl and Chlordimeform; the two extremes are presented to illustrate the maximum and minimum values for this Index. Again, if one excludes the Baygon flavor aversion data, the Behavioral Toxicity Indices are generally large, demonstrating the highly selective neurotoxicity of these insecticides and the relative sensitivity of these behavioral tests.

Table 1. Estimated ED50's for each behavioral test following acute exposure to various insecticides. Tests were ranked for each insecticide and the average ranking is shown at the bottom of each column.

Behavioral Test Insecticide	Figure-Eight Maze Activity	Radial-Arm Maze Performance	Schedule-Controlled Performance	Flavor Aversion
<u>Baygon</u>	2.0	3.7	3.4	indeterminant
<u>Carbaryl</u>	9.3	11.3	5.5	21.1
<u>Chlordimeform</u>	5.0	17.7	6.7	6.3
<u>Decamethrin</u>	7.6	3.7	3.5	1.6
Mean Rank of Sensitivity	$\bar{x} = 2.0$	$\bar{x} = 3.2$	$\bar{x} = 2.0$	$\bar{x} = 2.8$

Table 2. Estimated Behavioral Toxicity Index (LD50/ED50) determined for different behavioral measures following acute exposure to various insecticides. When two or more values for LD50 were available, the maximum and minimum values were used to calculate the Index.

Insecticide Behavioral Test	<u>Baygon</u> LD50 = 30 <sup>A</sup> mg/kg (I.P.)	<u>Carbaryl</u> LD50 = 200 <sup>A</sup> /48 <sup>B</sup> mg/kg (I.P.)	<u>Chlordimeform</u> LD50 = 300 <sup>C</sup> /123 <sup>D</sup> mg/kg (I.P.)	<u>Decamethrin</u> LD50 = 52 <sup>E</sup> mg/kg (P.O.)
Figure-Eight Maze	15	22/5	60/25	7
Schedule-Controlled Behavior	9	36/9	45/18	15
Radial-Arm Maze Performance	8	18/4	17/7	14
Flavor Aversion	Indeterminant	9/2	48/20	32

- A. Vandekar, et al., 1971.  
 B. B. WHO, 1971  
 C. Hollingworth, 1976.  
 D. Robinson et al., 1975  
 E. Kavlock et al., 1979

## DISCUSSION

These results convincingly demonstrate that behavioral measures provide sensitive diagnostic indices of toxicity. In general, these four behavioral tests were able to detect a highly specific behavioral toxicity of the various insecticides. The Behavioral Toxicity Index (LD50/ED50) provides an estimate of the comparative toxicity of these insecticides and ranged from 2-60. A large value for the Index reflects a relatively selective behavioral toxicity and indicates therefore that a behavioral change occurs at an exposure level substantially below that producing overt toxicity. The Behavioral Toxicity Index for Chlordimeform (based on the lowest reported LD50 of 123 mg/kg) for example, ranged from 7-25. For figure-eight maze activity, the ED50 was 5 mg/kg and yet no symptoms of toxicity such as ptosis were observed below 20 mg/kg. If similar data were obtained for a substance of unknown toxicity, they would strongly argue for further testing. This ability of behavioral tests to detect toxic effects at otherwise asymptomatic exposure levels supports their use as primary screens for neurotoxicity.

Across the limited range of insecticides tested, no behavioral measurement was uniquely sensitive to exposure. However, when the various tests were ranked for each insecticide and the overall ranking was computed, figure-eight maze activity and schedule-controlled performance were more sensitive than radial-arm-maze and flavor-aversion. Additional testing is needed to further characterize the flavor-aversion paradigm which was quite sensitive to Decamethrin exposure yet differentially sensitive to the two carbamates. We have no explanation for this latter finding.

Another dimension in assessing the utility of a behavioral test as a primary screen is its cost-effectiveness. If two tests are equally sensitive at detecting toxicity, the cost of performing the test will influence the final choice of methods. For behavioral testing, the cost will be influenced by several factors which include:

1. The equipment requirements which for the present array of tests ranged from water bottles to microprocessor-controlled test chambers;
2. The degree of automation which influences both the amount of technical support required for testing and the volume of testing which can be performed;
3. The amount of prior training of subjects which is required to establish a stable baseline of performance;
4. The variability of the behavioral endpoint which influence the number of subjects required to produce sufficient statistical power.

On the basis of these criteria, certain behavioral tests utilized in the present study appear more promising than others. For example, figure-eight maze activity and schedule-controlled performance appear to be equally sensitive in detecting the behavioral toxicity of these insecticides, yet cost considerations would favor the use of the figure-eight maze. Similarly, although flavor-aversion and radial-arm-maze performance require less initial training than does schedule-

controlled performance, the flavor-aversion paradigm fails to detect carbamate effects and the radial-arm-maze performance is less sensitive to Chlordimeform.

However, the utility of these behavioral measures has not been fully explored in this report. For example, data were not presented on the temporal distribution of locomotor activity and schedule-controlled performance or accuracy of selection with the radial-arm-maze. By the same token, parametric manipulations have not been made to optimize the sensitivity of each test. For example, introducing delays between selections in the radial-arm-maze is currently being explored as a way to increase the sensitivity of this procedure.

The Second Task Force for Research Planning in Environmental Health Science suggested that the selection of behavioral procedures for safety evaluation is optimized by testing the effects of known neurotoxicants on behavior. These data represent our first attempt to address this recommendation.

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#### ACKNOWLEDGEMENTS

The authors wish to thank the following individuals who offered essential aid in completing these experiments and preparing this report: Sharon Elliott, Winford A. Gordon, Glendora Heavner, Margaret Johnston, Phyllis Keeter, Karen Kidd and William Parrott.

PHARMACOLOGICAL PROBES AS A TOOL IN  
BEHAVIORAL TOXICOLOGY

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Delineation of the neurotoxic effects of various xenobiotic compounds is often complicated by the considerable compensatory capacity of the Central Nervous System (CNS). Moreover, the degree to which such compensation occurs may be critical when one is examining doses near the threshold for biological effect. The extent to which such compensation occurs is readily documented in the literature on recovery of function in the CNS following electrolytic or biochemical lesions.

The extent of recovery is a function of numerous factors including the size of the lesion and whether it occurred in single or multiple stages, recovery environment, sex, and age of the organism. The role of the latter variable is quite complex. Assessment of damage in the developing animal must balance greater CNS plasticity with greater vulnerability to insult. Compensation may result from a variety of mechanisms including redundancy (a part may carry out the functions of the whole), regeneration, multiple control (multiple localization), functional substitution, or a radical reorganization. Moreover, the organism may come to rely more heavily on information from previously ignored or under-utilized neuronal systems in decision-making. An excellent overview of this area has recently been published (Finger, 1978).

In spite of this compensatory capacity, deficits may appear when additional biological stress (e.g., illness, aging) is placed upon this "overtaxed" system. This tenet in essence underlies the use of pharmacological agents to unmask CNS insult. It is assumed that the compensated animal is operating with compromised neurochemical substrates and thus cannot respond to this drug challenge in the same manner and/or degree as normal animals.

The use of pharmacological probes as physiological stressors has a number of advantages. It is a noninvasive, reversible procedure that can be introduced at any time point in the life of the animal. Moreover, the pattern that may emerge from the selective administration of the various agonists, antagonists, depletors, etc., can provide insight into the nature of the "affected" neurochemical substrates. Judicious selection may even allow one to discriminate between peripheral versus centrally-mediated effects. Furthermore, negative results imply that a number of defined systems are functioning normally.

I will provide some examples of recent studies employing pharmacological probes. I will also describe how we have attempted to extend these efforts in utilizing a drug-discrimination learning paradigm. This paradigm has been well validated in psychopharmacology as a tool for the assessment of drug-response thresholds.

I have restricted the text along two avenues. For one, only studies employing behavior as an endpoint have been cited. This does not, however, obviate the use of this technique if one desires to assess the functional capacity of other target organs. I have also excluded the wealth of studies that has assessed the impact of simultaneous chronic exposure to an environmental compound and a drug. These studies, directed at related questions, obviously provide a historic precedent for the incorporation of drug challenges into current screening protocols.

Following a report by Fouts et al. (1963) that DDT, chlordane, and other analogues produced a decrease in hexobarbital-induced sleep time, numerous other investigators have used this variable as an end-point to examine the effects of various environmental agents on liver and/or kidney function. In the area of neurobehavioral toxicology, assessment of CNS status with drug probes is fairly recent. However, this technique has now been widely used with a variety of environmental compounds, tested in different behavioral tasks, and examined in young and adult animals that had either been previously on or were concurrently being exposed to the toxin at the time of testing.

Pertinent to the population of major concern for this audience, namely occupational exposure, are investigations employing drug challenges in animals exposed only as adults. In an investigation by Tilson et al. (1979), adult rats were exposed to 589 ppm of carbon disulfide ( $\text{CS}_2$ ) in inhalation chambers. Animals were tested on a number of neuromotor tasks. Spontaneous locomotor activity was also assessed in Automex activity monitors (Columbus Instruments). One and three weeks after exposure had terminated, the rats received a 3 mg/kg injection of d-amphetamine sulfate and were subsequently tested over the next 80 minutes. Analyses of the data revealed no differences among the groups in terms of baseline motor activity. However, one week after treatment had ceased, the  $\text{CS}_2$  animals exhibited an attenuated increase in activity relative to the controls following drug injection (Figure 1). These differences had disappeared by three weeks post-exposure.

Rafales et al. (1980) have examined the response to drug challenge both during and following exposure to acrylamide. Ninety-day old male rats were exposed to 100 ppm of acrylamide via drinking water. A variety of behavioral tasks were conducted, including the measurement of locomotor activity in photocell chambers. Animals were tested three times a week. Following a one-hour baseline period, animals were injected with 1 mg/kg (s.c.) of d-amphetamine sulfate and then monitored for two additional hours. This procedure was followed for four weeks prior to exposure, during the six weeks of exposure, as well as 12 weeks post-exposure. Results of the study



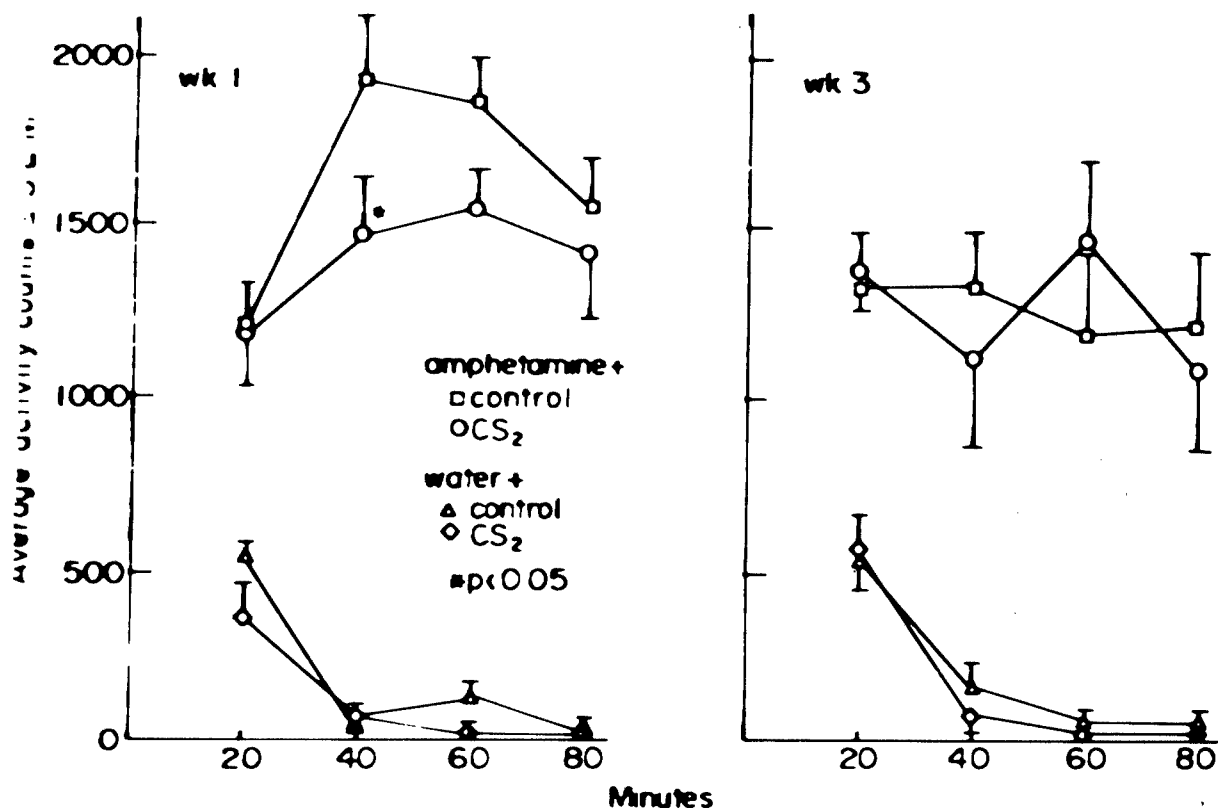


Figure 1. Average activity counts  $\pm$  SEM prior to (water) and following a 3 mg/kg injection of d-amphetamine sulfate. Animals were tested one and three weeks following exposure to 589 ppm CS<sub>2</sub>. (Tilson et al., 1979)

are graphed in Figure 2. No shifts were observed in baseline activity either during or following exposure to acrylamide. Moreover, in all phases of the study (pre-exposure, exposure, recovery) amphetamine treatment produced a significant increase in activity during both the first and second hours post-injection. However, the animal's activity levels were significantly higher during the exposure and recovery phases relative to the pre-exposure period. More specifically, by the end of the six week exposure period, activity in the first hour had significantly increased over the baseline drug response and remained elevated until the seventh week of recovery. The effect of acrylamide was even more apparent and persistent in the second hour. This activity was elevated above the second hour, pre-exposure drug response for each of the last four weeks of the exposure period and remained so until the tenth week of recovery. Data from this study exemplify the utility and power of pharmacological probes to disclose persistent neurobehavioral alterations in individuals that have been removed from the exposure setting for a substantial period of time.

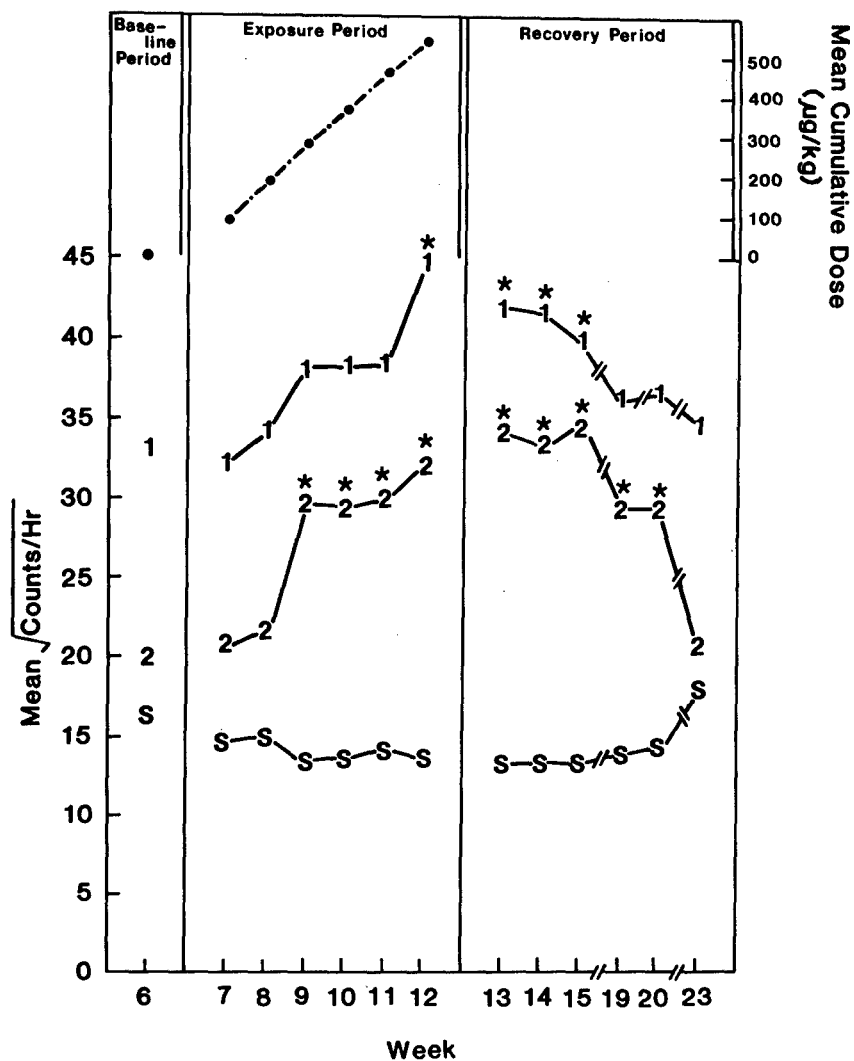


Figure 2. Locomotor activity in rats following a 1 mg/kg injection of d-amphetamine sulfate. S represents activity one hour prior to injection; 1 and 2 equal activity levels one and two hours post-injection. (Rafales et al., 1980)

In an interesting reversal of protocol, Thomas et al. (1979) have examined the effects of microwave radiation on operant performance in rats concurrently receiving chlordiazepoxide (Librium), a common tranquilizer. This type of manipulation examines questions along a somewhat different vein from those addressed above. However, the study of such interactions is highly relevant to the occupational setting since a substantial proportion of the

industrial work force engages in drug-taking behavior, prescribed or otherwise. In this investigation, rats were trained to press a lever on a fixed-interval schedule of reinforcement (FI). In essence, the first lever press after one minute produced a food pellet. Intermittent responses had no consequences but were recorded. After the behavior was stabilized, a dose-effect function was established for each animal over a dose range of 1-40 mg/kg (i.p.) chlordiazepoxide HCl. Injections occurred 30 minutes prior to testing. This phase of the experiment was then repeated except the animals were concurrently exposed to a  $1\text{mW/cm}^2$  of microwave radiation. This level is relatively low intensity and typical of many radar sources. Animals had previously been adapted to a special restrainer so that this manipulation would not affect FI performance. The results of the study are illustrated in Figure 3. Increasing doses up to approximately 10 mg/kg of chlordiazepoxide alone increased FI response rates; further increases in dose decreased rate. In combination with microwave irradiation the shape of the curves remained relatively constant but the magnitude of the effect was enhanced. It should be pointed out that this synergism is not particularly beneficial to the animal in this task and in fact is a rather wasteful expenditure of energy. On the FI schedule, delivery of reinforcement is timelocked and independent of response rate. Elevated responding does not alter reinforcement probability as is true of ratio schedules of reinforcement. The importance of additional investigation along the lines of the Thomas et al. study cannot be overstated. The interaction between drug intake and exposure to chemicals in the work environment has relevance not only in terms of job performance but also with regard to the potential altering of thresholds for toxic health effects.

The drug challenge technique has also been used to study animals exposed to xenobiotics during early development. Hughes and Sparber (1978) imposed a drug challenge (d-amphetamine) on an ongoing operant task to disclose postnatal consequences of in utero exposure to methylmercury. In that study, pregnant rats were administered varying doses of methylmercury dicyandiamide via gavage on either days 0, 7 or 14 of gestation. At six months of age, offspring were trained, under an autoshaping procedure, to press a retractable lever to obtain a food pellet reinforcement. Following acquisition of the bar pressing behavior, the animals received daily sessions consisting of 32 trials in which the number of responses emitted and latency to make contact with the lever were recorded. Thirty responses in a session for five sessions was set as the criterion prior to drug challenge. Analyses of these baseline data revealed no differences in performance as a function of treatment history. The drug challenge consisted of each animal receiving 0, 0.5, 1.25, 2.5 or 3.75 mg/kg of d-amphetamine sulfate prior to the start of a test session. Exemplary of the data are the results seen in male offspring of dams gavaged on day 0 of gestation. As seen in Figure 4, the high dose of d-amphetamine failed to disrupt the rate of response or latency to respond as markedly in the methylmercury progeny relative to their control counterparts. Two aspects of this study are worth emphasizing. One, the utilization of a drug probe disclosed a difference that would have been missed if one had simply relied upon baseline data. Two, this technique revealed an effect late postnatally, many months removed from the initial exposure period (i.e., gestation).

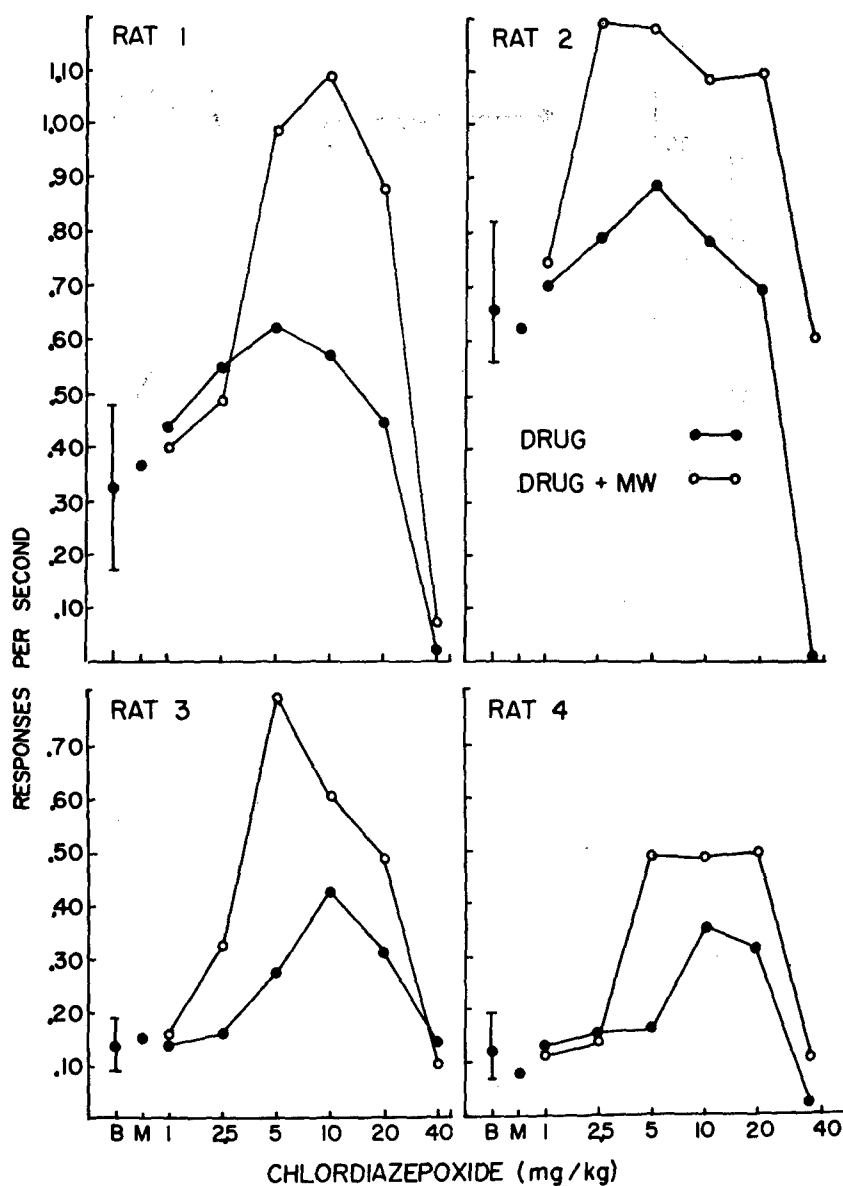


Figure 3. Number of bar press responses/sec on a fixed interval schedule following injections of chlor diazepoxide alone or with the concurrent exposure to 1mV/cm of microwave radiation. (Thomas et al., 1979)

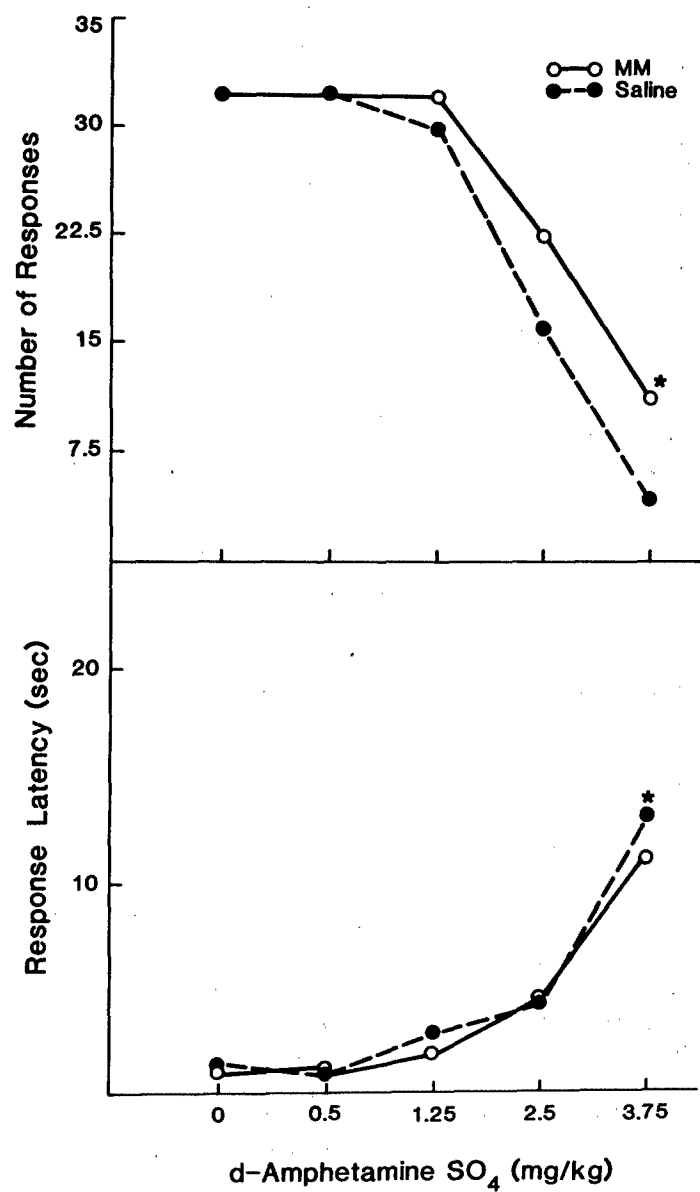


Figure 4. Performance of 6 month old rats in an operant bar press task following d-amphetamine injections. Contrast is between control offspring and offspring from dams treated with methylmercury on day 1 of birth. (Redrawn from Hughes and Sparber, 1978)

Pharmacologic probes have been most extensively applied in the investigation of lead neurotoxicity. Two of these studies will be detailed. These investigations employed similar exposure protocols, but different species, tasks, and drug challenges.

Mailman et al. (1979) administered lead (100 mg/kg) via intubation to rats on days 3 through 30 of life. Subsequently, at periodic intervals up to six months of age, animals were injected with lithium (2 nmoles/kg; 4 M LiCl; i.p.) and water consumption monitored. Such treatment has been shown to induce polydipsia in rats. Although there were no differences in baseline water consumption, administration of lithium caused lead-treated rats to become significantly more polydipsic, increasing their fluid intake to nearly three times more than the controls (Figure 5). The same trend was also exhibited by lead-treated female rats. The persistence of this enhanced-polydipsia suggests that a permanent alteration in neural substrate accompanied the neonatal lead exposure.

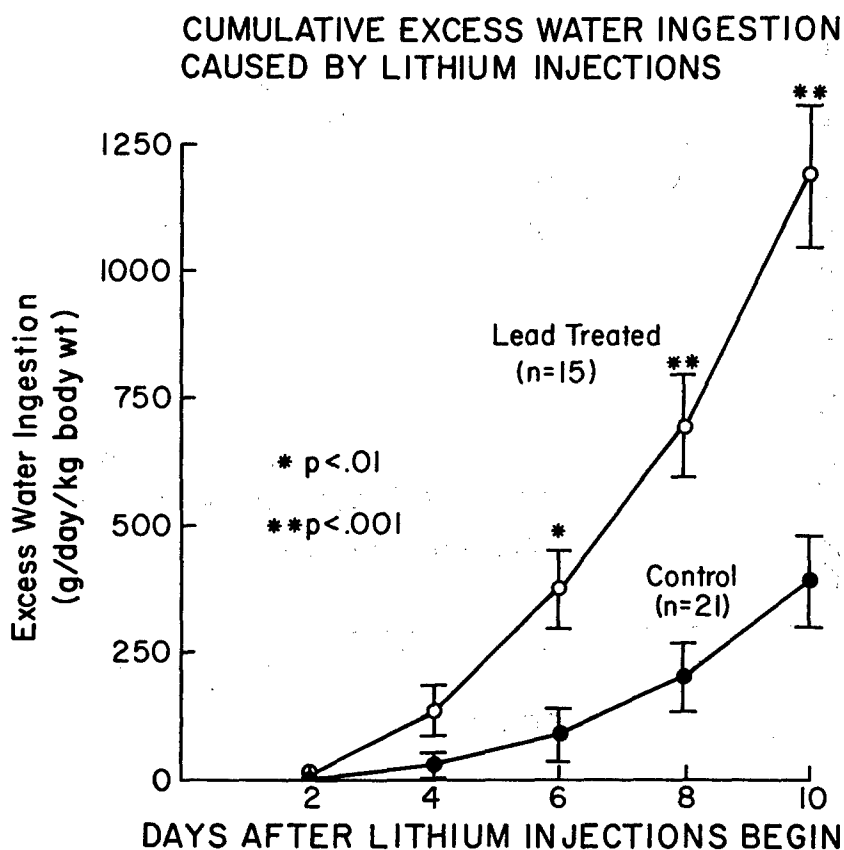


Figure 5. Contrast of lithium-induced polydipsia between controls and pups intubated with lead acetate between days 3 and 30 of lactation. Rats were tested periodically up to 6 months of age. (Mailman et al., 1978)

In a study varying the length of lead treatment, Rafeles et al. (1979) examined locomotor activity in adult mice exposed to lead only during lactation or during lactation and continued post-weaning. At 140 days of age, spontaneous activity was recorded for a three hour period over five consecutive days. During the fifth session, d-amphetamine sulfate (5 mg/kg; i.p.) was administered. As seen in Figure 6, the lead groups did not differ from controls in terms of baseline spontaneous activity. However, drug administration resulted in a significantly different alteration in activity in the lead groups relative to the controls. More important is the fact that the direction of this alteration was a function of lead history. Animals exposed only during lactation were hyperactive relative to controls, whereas the offspring with lifetime histories of lead exposure were hypoactive (Figure 7).

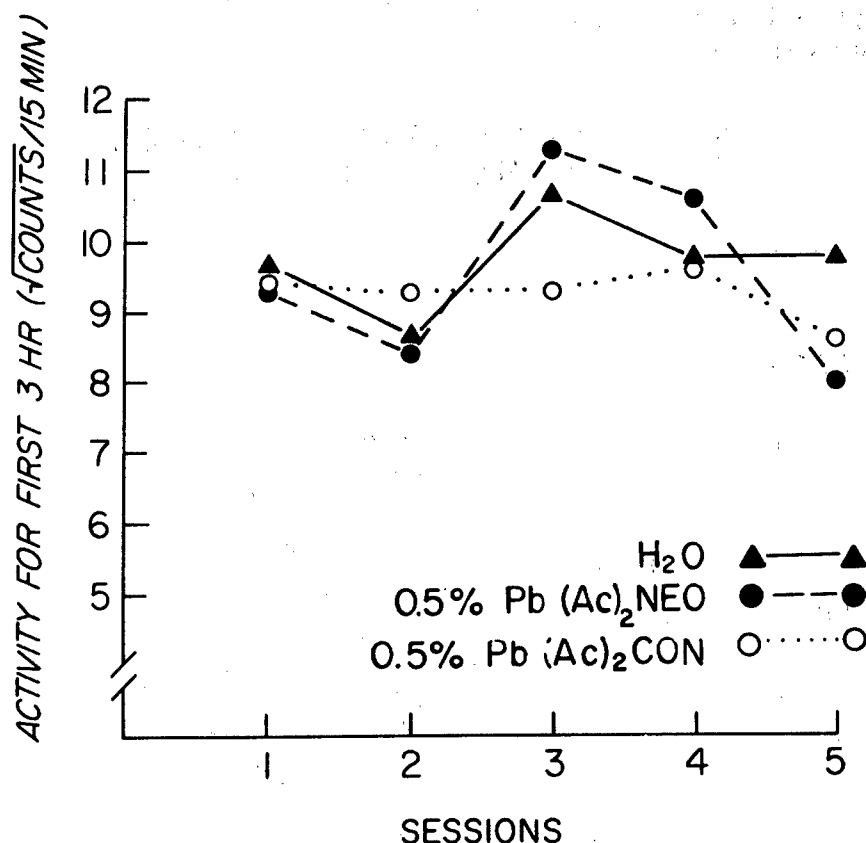


Figure 6. Baseline (1 hr. session) activity levels in controls and offspring exposed to .5% lead acetate from birth (Con) or only during lactation (Neo). (Rafeles et al., 1979)

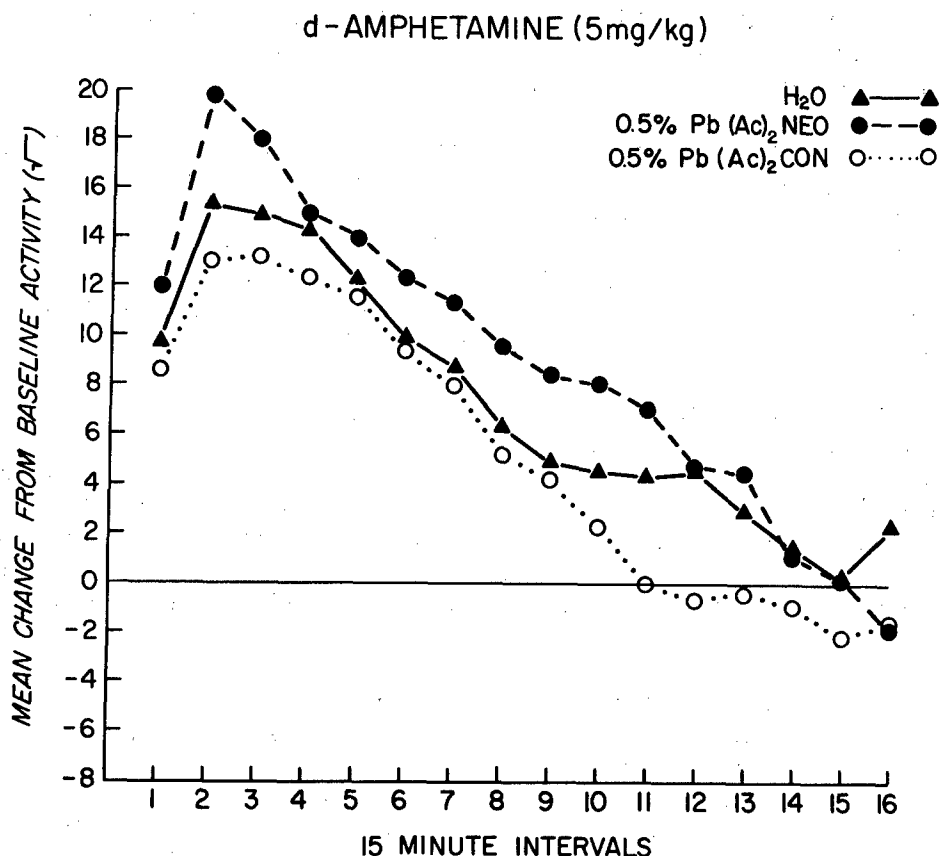


Figure 7. Changes in locomotor activity of controls and lead-offspring to a 1 mg/kg injection of d-amphetamine sulfate. Group designation same as in Figure 6. (Rafales et al., 1979)

The utility of the drug challenge technique is again substantiated by these data in that it allows one to distinguish between two treatment populations, wherein baseline data would not have disclosed these differences. The results also suggest that continued postweaning Pb exposure does not result in a magnification of a deficit produced during early development but rather produces a different picture of CNS insult.

In addition to these two studies, numerous other investigators have documented this altered drug-responsiveness following neonatal lead exposure. However, the direction of the drug response relative to controls has been variable and appears to be the result of a myriad of factors including the nature of the task, dose, and duration of response. As a result, conflicting theories of either a hyposensitive or hypersensitive catecholaminergic system have been postulated.



To achieve better clarification, we have utilized a drug-stimulus discrimination paradigm to address this question. This paradigm is frequently employed in psychopharmacology and has been validated as a tool for assessing drug-response thresholds (Ho et al., 1978). The task is analogous to a simultaneous discrimination problem with the only cue for directing response selection being the endogenous drug state, i.e. whether the animal has received a drug or "no-drug" (vehicle) injection. We hypothesized that if the Pb-exposed animal was hyposensitive to amphetamine then it would have an elevated response threshold relative to the controls in terms of the lowest dose capable of cueing the animal to emit the response appropriate to the "drug" condition. On the other hand, hypersensitivity would shift the drug discriminability threshold to below that of the controls.

Twelve female Long-Evans hooded rats (Charles River) were mated at 90 days of age and then subsequently exposed to either 0, 0.02, or 0.5% Pb acetate in their drinking water (4 dams/group) within 12 hours after parturition and for the duration of lactation. At weaning (day 21), two males were randomly selected from each of the Pb-exposed litters with one male pup placed on the Pb treatment of its dam while the other male pup was placed on distilled water. In addition, one male was randomly selected from each of the four control litters and placed on distilled water.

At 90 days of age, the animals were gradually reduced to 80 percent of their ad lib weight. Animals were then trained to bar press in a two-bar Colbourne operant chamber with reinforcement available on one lever following drug injection and on the "no-drug" bar following saline injection. Reinforcement was 0.1 ml of 33 percent condensed milk solution with animals shaped to a fixed ratio 10 (FR 10) maintenance schedule. The training dose, 1 mg/kg of d-amphetamine sulfate (s.c.), was injected 30 min. prior to testing followed by a 30 min. test session. An animal was judged to have accurately made the discrimination between saline and amphetamine injection if, on the first 15 bar presses of a session, five or fewer presses were emitted on the incorrect bar prior to the completion of 10 presses on the correct bar. If this criterion was met on eight out of 10 consecutive sessions, then the animal's drug dose was lowered by approximately 30 percent. Whenever criterion was not attained on eight out of 10 consecutive sessions, the dose was raised by 30 percent for the next 10 sessions. This lowering and elevating of dose was done over successive 10-session blocks with testing terminated when the animals failed to meet criterion when tested twice (10 session blocks) at a particular dose.

Discrimination data were analyzed in terms of the lowest dose preceding each animal's initial failure to meet the criterion on eight out of 10 consecutive sessions as well as the dose where the final threshold level was set. Analyses revealed significant treatment effects in that the controls had significantly lower drug thresholds ( $M \pm SD = .14 \pm .07$ ) than either Groups .02/0 ( $.29 \pm .04$ ) or Group 0.5/0 ( $.26 \pm .05$ ) or than the two groups continued on Pb after weaning, namely Groups .02/.02 ( $.23 \pm .04$ ) and 0.5/0.5 ( $.3 \pm .04$ ) (9). There were no significant differences between original and final thresholds or any significant interactions.

The dose response curve for each treatment group during initial training is graphed in Figure 8 and reflects the number of animals in each group meeting criterion eight out of 10 consecutive sessions at each dose level. Furthermore, the performance for each group at each dose is also indicated (in parentheses) as the mean percent of trials within a 10-block session at which criterion was met. As can be seen from this figure, the retention of more control animals at the lower doses is reflective of their superior discriminative ability.

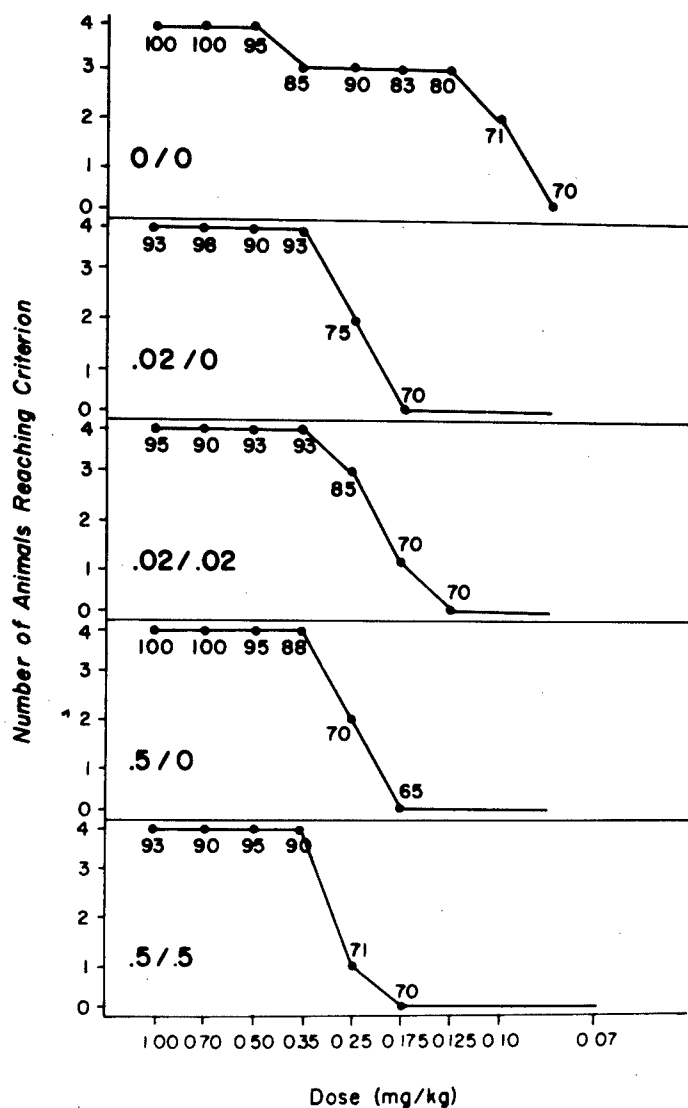


Figure 8. Number of animals in each treatment group reaching criterion during original training. Numbers on lines represent the group mean percent of trials that criterion was met at each dose. Group designations described in text. (Zenick and Goldsmith, 1980)

These data substantiate the hypothesis of an amphetamine-hyposensitivity in the Pb animal. Equally important is the observation that this alteration appears to be dependent upon early, neonatal exposure since the deficit was seen in all Pb-exposed animals whether or not they were continued postnatally on Pb.

There are several lines of evidence that suggest that this alteration may, in part, reflect an alteration in the dopaminergic system. Indirect support for this contention is provided by data from drug-discrimination studies that have attempted to delineate the neurochemical substrate of the amphetamine cue. Schechter and Cook (1975) employed a number of specific biogenic amine depletors (e.g.,  $\alpha$ MPT, disulfiram) and selective receptor antagonists (e.g., haloperidol, phenoxybenzamine) and observed their effects on the discriminative properties of amphetamine. Their data suggested that dopaminergic systems mediate the interoceptive cue produced by amphetamine. Similar findings have been reported by others.

Conclusions regarding the neurochemical effects of Pb toxicity are not as clear-cut. In various articles, Pb has been reported to affect many of the putative neurotransmitter systems. Thus, theories abound regarding the neurochemical substrate involved in Pb toxicity. However, the results of the present study suggest that with the appropriate manipulation of antagonists, depletors, and agonists, the drug-discrimination paradigm can provide insight into the neurochemical basis of the elevated drug thresholds observed in the Pb animals in this study.

Because of the time required to conduct a drug-discrimination study (minimum three-six months), this procedure should be employed in later phases of drug-probe investigations. Positive results on less time-consuming tasks (e.g., locomotor activity, spontaneous alteration, etc.) should precede such studies. However, the eventual incorporation of the drug-discrimination paradigm can then serve to better pinpoint "affected systems" for subsequent neurochemical investigations.

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BEHAVIORAL CONSEQUENCES OF IN UTERO  
EXPOSURE TO TOXIC AGENTS

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INTRODUCTION

Damage to the human fetus during gestation has long been recognized, and the description of some terata is as old as human history. Skeletal malformations and alterations of overlying soft tissue are most easily recognized. Alterations of deeper tissues are less readily detected. Functional alterations may be unnoticed unless specific tests are used.

The classic teratogenic study which detects morphological alterations in experimental animals, particularly rats, is well known. Recently attention has been focused on the teratogenic potential of numerous toxic chemicals in the environment. Exposure on the fetus can occur from maternal inhalation, ingestion, or application to the skin. While many chemicals of interest are man-made, or are the result of intentional addition of the chemical to the environment by man, some exposures result from chemicals which are not added by man to his environment. The consequences of exposure of the human fetus to toxic agents may vary from the well-recognized skeletal malformations resulting from thalidomide, to the damage to sensory systems from rubella virus and the more generalized brain damage caused by in utero exposure to methyl mercury.

Having recognized these gross defects, it has been proposed that we should look for functional disorders as the more subtle consequences of exposure to toxic agents, particularly disordered behavior. This proposal has not been unproductive since it has called attention to the magnitude of the problem of teratogenesis, even resulting in the coining of the title "behavioral teratogenesis". There seems to be little reason not to expect that "endocrinological teratogenesis" or "immunological teratogenesis" can also be found. While it is important to realize that disordered function of a system can be a significant consequence of gestational exposure to a toxic agent, it does not follow that functional changes will be present consistently with conditions of exposure below those which produce morphological damage. That is, functional changes may not be the most sensitive indicators of damage to an organ system. In fact, it can be stated confidently that morphological

damage to the central nervous system sometimes occurs before functional changes are detected (Norton, 1978). Among other reasons, the adaptability of the central nervous system (CNS) and the numerical redundancy of some types of neurons combine to diminish the functional consequences of neuronal loss. Thus the limits of behavioral teratogenesis need to be explored for each type of exposure.

#### BEHAVIORAL CONSEQUENCES OF GESTATIONAL EXPOSURE

In studying the effects of gestational exposure to toxic agents on behavior, the duration and intensity of exposure are critical in determining the outcome, as they are in toxic consequences in adult animals. A third factor is unique to gestational exposures, the stage of development of the fetus. Markedly different behavioral consequences can be obtained by as little as 24 hour difference in time of exposure during gestation in the rat (Norton et al., 1976). Just as the morphologic effects of toxic agents can be related to the time of exposure of the developing nervous system, so the behavioral changes can be identified with certain exposure periods. In mammals the CNS is laid down at an early stage of organogenesis as a simple tube, but the later stages of neuronal multiplication, migration, and differentiation last into postnatal life. Because the CNS develops and enlarges in a rostral direction in a fixed growth sequence of interdependent areas, interference with cell division or migration at a specific period in development is known to leave a permanent and characteristic scar in the morphologic organization. It is possible to use this specificity to begin to develop a behavioral timetable which identifies, at least for the rodent, the time of maximum effect on a particular behavior. For example, X-irradiation on gestational day 14 in the rat causes more hyperactivity in the adult than irradiation on day 16. Thus, a standard dose of irradiation has different long-term behavioral effects when given on different gestational days (Norton et al., 1976).

The major period of organogenesis in the rat ends at about the 14th day of gestation, where the day on which the female rat is sperm positive is considered the first day of gestation. From gestational day 14 to the end of gestation, and into the first few postnatal weeks, the cellular components of the CNS are increasing, migrating, and differentiating rapidly. This is a period of vulnerability of the cells to various types of insults. It may be expected that behavioral teratogenesis may result from CNS damage by exposure to a toxic agent at this late period of gestation without major damage to other organ systems. Since the CNS develops in a generally rostral direction, the vulnerability of the telencephalon occurs latest in development, even into the postnatal period. Selected cell populations in other brain areas also have a late period of vulnerability, notably the small granule cells in the hippocampus and cerebellum.

The unique organization of the CNS makes analysis of the relation of structure to function very difficult. It is not possible to predict in detail the behavioral consequences of many types of CNS damage. Even so, the different

types of interference with CNS development which accompany exposure to toxic agents add importance to the study of these agents. Not only are the consequences of experimental exposure of the fetus useful in predicting danger to humans, but also the potential exists for increased understanding of the relation of structure to the function by studying the behavioral consequences of damage to different anatomical areas. In this, the most useful developmental period is from the end of major organogenesis to the end of glial proliferation and neuronal differentiation in the early postnatal period.

The behavioral consequence of exposure of the fetus has the fundamental dose response characteristics of toxic exposures in general. Higher doses tend to increase the amount of behavioral damage. This principle is not different from the result of the classic experiment showing that removal of more and more cerebral cortex in the rat causes progressively more disturbance of maze-running ability.

Several batteries of tests have been proposed for the evaluation of early postnatal behavior after gestational exposure to toxic substances, such as the battery described by Vorhees and colleagues (1979). The behavioral batteries evaluate the development of various reflexes and complex behaviors and usually include time of appearance of developmental landmarks such as eye opening. A simple battery is given in Table 1. It is important that these early postnatal tests are administered in ways which detect the time of onset of a particular simple or complex behavior. For example, the auditory startle response is just present at about postnatal day 12 (Culver and Norton, 1976) and the passive avoidance response is first acquired at about day 15 in rats (Schulenberg et al., 1971).

TABLE 1. EVALUATION OF BEHAVIORAL TERATOGENESIS

Behavior tested	Test method	References
1. Sensory development/function	Auditory startle response Eye opening	Fleshler, 1965; Culver and Norton, 1976
2. Visual-motor coordination	Surface righting Reflex suspension	Altman and Sudarshan, 1975; Barlow et al., 1978; Brunner et al., 1978
3. Locomotor activity	Open field exploration	Walsh and Cummins, 1976
4. Perseveration/habituation	Olton-type maze Repeated maze exploration	Norton, 1976; Olton et al., 1978
5. Conditioned acquisition/retention	Passive avoidance	Schulenberg et al., 1971; Riley et al., 1979

The results of the application of test batteries to early development of behavior after exposure to a toxic substance have often been somewhat disappointing. It may seem intuitively correct that behavior should be a subtle way of detecting damage in the absence of morphologic abnormalities and this doctrine has been proposed in various recent publications (see, for example, Spyker, 1975). However, the postnatal behavioral consequences of brain damage to the fetus are complex. The following argument presents an attempt at categorizing some of these consequences and relating them to the type of damage caused by toxic agents.

There are several ways in which toxic substances affect the fetus in reproductive studies (Table 2). First, the prenatal genetics may be damaged if the adults are exposed prior to mating, resulting in transmission of the genetic change to the chromosomal make-up of the offspring. In addition, toxic substances may alter the maternal environment either by genetic effects on the mother, in which case the toxic substance need not be present during gestation, or by being present during gestation and altering the environment in which the fetus develops. In the latter case, the damage may be directly to the fetus or indirectly through toxicity to the mother. This concept is elaborated in Figure 1. The figure shows three types of fetal damage resulting from the action of toxic agents on the maternal environment.

TABLE 2. SOURCES OF DAMAGE TO FETAL CNS

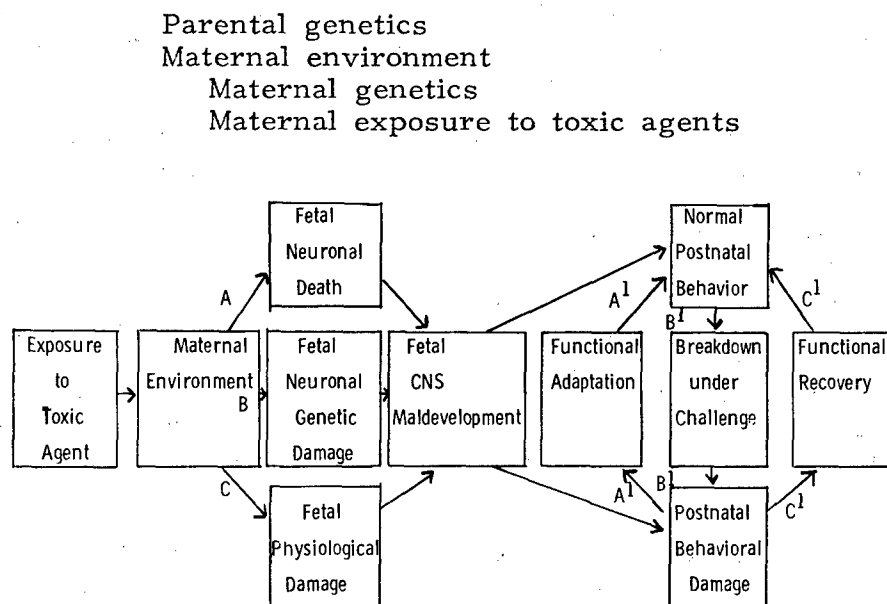


Figure 1. Three categories of fetal CNS damage (A, fetal neuronal death; B, fetal neuronal genetic damage; C, fetal physiological damage) and three paired behavioral consequences (A<sup>1</sup>, postnatal behavioral damage followed by functional damage adaptation and normal postnatal behavior; B<sup>1</sup>, normal postnatal behavior followed by breakdown under challenge and postnatal behavioral damage; C<sup>1</sup>, postnatal behavioral damage followed by functional recovery and normal postnatal behavior).



## TYPES OF GESTATIONAL DAMAGE

There may be a loss of neurons in the CNS. Cells undergoing mitosis or other cells with unique metabolic demands may be particularly susceptible. Death of germinal cells in the ependymal lining of the ventricles will have long term consequences on the cell population of the brain. Reduction in the number of germinal cells may reduce the total population of neurons and may alter the morphology in other ways, since the brain is built on a sequential plan and loss of one set of dividing cells may interfere with subsequent migration of new waves of cells. Thus, the complex structure of the brain makes it difficult for repair processes to restore the developmental pattern to normal after loss of one wave of cell division. Carbon monoxide is an example of an agent which causes death of cells in the ependymal layer through hypoxia. An example of the permanent morphologic consequences of hypoxia of the ependymal layer is the production of ectopia of the caudate nucleus (Daughtrey and Norton, 1980).

Some agents may cause chromosomal damage, not all of which results in cell death. Cells with chromosomal damage may survive but function inadequately. X-irradiation is an example of an agent which has the potential for causing chromosomal damage at levels which do not kill all dividing cells. There is evidence that neurons in animals subjected to gestational irradiation have smaller nuclei than normal and tend to show early degenerative changes in postnatal development (Norton, 1979; Schneider and Norton, 1980). While this is not direct evidence of chromosomal damage, it is evidence that cells which have not been killed by X-irradiation may be functionally abnormal.

Another type of toxic effect on the neuron is proposed in the category, fetal physiological damage, in Figure 1. An example is the condition of hypothyroidism which may be induced in the developing fetus by various agents. The thyroid gland develops functionally about gestational day 17 in the rodent (Theiler, 1972). Although early exposure of the fetus to goitrogens does not result in marked morphologic changes in the CNS, the effect of loss of thyroid hormones during the period of neuronal differentiation is dramatic. Thyroid hormones are essential in the late fetal and early postnatal periods for the normal formation and elaboration of neuronal processes. Reduced dendritic branching is characteristic of neonatal rats that have been exposed to antithyroid chemicals during the perinatal period (Comer and Norton, 1980).

The three types of damage just discussed, neuronal death, neuronal genetic damage, and fetal physiological damage, are listed in Table 3 with an example of a toxic agent which causes each type of damage.

TABLE 3. TYPES OF DAMAGE TO FETAL CNS  
(Letters A, B and C refer to Figure 1)

Damage	Example
A. Fetal Neuronal Death	Hypoxia from CO
B. Fetal Neuronal Genetic Damage	X-irradiation
C. Fetal Physiological Damage	Antithyroid chemicals

Figure 1 categorizes the behavioral consequences of the type of CNS damage just described into the two obvious possibilities, normal or abnormal behavior. Normal behavior may result in the presence of CNS damage if the redundancy of the cell complement of the CNS has not been exceeded by the damage or if the functional adaptation of the CNS is adequate to restore normal function. If either of these two protective mechanisms is exceeded, behavioral alteration will result. In the analysis of behavior after gestational exposure there is an additional complication, which is the long-term consequence of neuronal cell damage. The immature brain may show functional alterations for which the mature brain can compensate, since the adaptive process may continue over a prolonged postnatal period. An example of this is suggested in Table 4 where neuronal cell death in the fetal period results initially in behavioral damage seen as juvenile hyperactivity and followed by recovery in the adult. This condition has been suggested as a possible consequence of hypoxia in the perinatal period in humans and has been demonstrated in the neonatal rat exposed to carbon monoxide. When 5-day-old rats were exposed to carbon monoxide to the point of severe respiratory distress over a period of 1 to 2 hours, the rats developed early hyperactivity which persisted until they were 3 months old, followed by complete recovery from the hyperactivity (Culver and Norton, 1976).

TABLE 4. PREDICTED BEHAVIORAL CONSEQUENCES  
(Letters A<sup>1</sup>, B<sup>1</sup> and C<sup>1</sup> refer to Figure 1)

- A<sup>1</sup> Fetal neuronal cell death → behavioral adaptation with early functional recovery ("juvenile hyperactivity")
- B<sup>1</sup> Fetal neuronal genetic damage → chronic functional abnormality and/or mature breakdown of adaptation ("early senility")
- C<sup>1</sup> Fetal neuronal cell differentiation delay (e.g. hypothyroidism) → marked developmental delay followed by some functional recovery

The consequences of damage to the genetic material of the neuron may result in a cell which is able to survive but which may not be able to respond adequately to functional demands. Such a cell would be predicted to respond poorly to challenge; for example, functional challenge from administration of psychoactive drugs. In addition, cells with irreparable DNA damage might well show an early type of senility; that is, the neuron might function adequately at first in the young adult animal but might not continue to function normally throughout life and might show early degenerative changes. Evidence from study of the consequences of gestational irradiation supports these possibilities. It has been shown that young rats irradiated during gestation (125 R on gestational day 15) are not hyperactive compared with control rats. However, the irradiated rats overreact to the administration of low doses of morphine sulfate (2 mg/kg, s.c.) by becoming more hyperactive than control rats receiving the same dose of drug (Schneider and Norton, 1979). The same parameters of X-irradiation also result in excessive accumulations of dendritic varicosities and loss of dendritic spines on cortical pyramidal neurons in mature rats (about 6 months old) (Norton, 1979). These changes are typical of degenerative changes in neurons under a variety of conditions (see, for example, Bernstein et al., 1975; Scheibel et al., 1974). Along with morphologic

signs of degenerative changes in the CNS, gestationally irradiated rats become progressively more hyperactive as they mature (Norton, 1979).

Two types of damage have been discussed: interference with neuronal cell production and genetic damage caused by toxic substances. The third category of toxic effect occurs after production of neurons has essentially ceased. At this time the immature neuron still must undergo differentiation to form the mature neuron with all its processes and synaptic connections. At this point toxic agents may interfere with normal differentiation, resulting in maturational delay. Extreme malnutrition has been reported to cause maturational delay. However, the classic cause is hypothyroidism. An extensive number of agents have been reported to depress the thyroid of the developing animal. The critical period for exposure to goitrogens is in the perinatal period when the presence of thyroxine is required for normal neuronal differentiation. Table 5 contains a partial list of agents known to induce hypothyroidism.

TABLE 5. ENVIRONMENTAL ANTITHYROID AGENTS

Goitrogen	References
Thiocyanates	Ekpechi et al., 1966
Oxalic Acid	Goldman and Doering, 1979
Anions ( $I^-$ , $Br^-$ , $NO_3^-$ , $ClO_4^-$ )	Barker, 1936; Langer and Greer, 1977; Sternthal et al., 1980
PBB's	Wastelle et al., 1978; Bahn et al., 1980
DDT, DDE	Naber, 1977; Ringer and Polin, 1977
Pb, As, Co	Underwood, 1973; Langer and Greer, 1977
Fetal Ethanol	Kornguth et al., 1979
Lithium	Langer and Greer, 1977

Environmental exposure to most of these agents may occur. The behavioral consequence of hypothyroidism is maturational delay which parallels the delay in neuronal differentiation. Table 6 gives a brief comparison of three developmental behaviors in control and in rats made hypothyroid by administration of methimazole to the mother from day 17 of gestation to postnatal day 10. These data are taken from a report by Comer and Norton (1980). The extent of the developmental delay is very marked in these experiments. It seems appropriate to suggest that whenever marked developmental delay occurs as a result of gestational exposure to a toxic substance, an effect on the thyroid should be suspected.

TABLE 6. BEHAVIORAL DEVELOPMENT IN  
HYPOTHYROIDISM IN RATS

Behavioral Test	Postnatal Day of Onset or Duration of Response	
	Control	Hypothyroid
Onset of Auditory Startle Response	Day 12	Day 17
Onset of Eye Opening	Day 12	Day 18
Duration of Reflex Suspension (Tested Day 14)	~ 30 Sec	~ 5 Sec

### CONCLUSIONS

Some types of damage to the fetal CNS which can occur late in gestation, after major organogenesis, have been described. The types of damage are described as consequences of a) hypoxia, b) damage to DNA, and c) suppression of thyroid hormones. Characteristic and different behavioral patterns emerge in each of these conditions.

Hypoxia causes focal cell death, probably in areas of unusual metabolic vulnerability. Hyperactivity in the young animal is a behavioral consequence and recovery may occur with maturation. Various agents may cause fetal hypoxia, including agents which depress maternal respiration, oxygen-carrying capacity of the blood, maternal blood pressure, or cell respiration.

Damage to the genetic material in surviving neurons, presumed to result from various agents which interfere with DNA replication, such as X-irradiation, has little initial behavioral consequence unless the animal is challenged. Maturity signals an excessive level of neuronal degeneration and the development of hyperactivity. This argument predicts that gestational exposure to hydroxyurea, for example, would resemble X-irradiation in causing little behavioral damage in early postnatal life. This predicted result agrees with the report on hydroxyurea by Vorhees and colleagues (1979).

The behavioral pattern of effect of hypothyroidism is marked developmental delay, which is not associated with exposure to the other types of agents, followed by considerable recovery if the goitrogen is removed early in postnatal life. Various agents, some of which are listed in Table 5, can cause hypothyroidism. Some behavioral development tests are shown in Table 6.

The three categories of damage are summarized in Table 7. It is important to emphasize that this classification is tentative and needs to be reviewed and expanded. It is not intended to describe in any detail the various behavioral alterations which might be demonstrated by application of a series of behavioral tests. Nor is it intended to account for all of the unique chemicals

which may cause toxicity limited to damage to a single cell type or special biochemical mechanism. Rather, it is intended to point out that agents may cause fetal hypoxia by a variety of means; many agents which are recognized to be fetotoxic can damage DNA; and more compounds suppress the thyroid than the classic goitrogens. It may be argued that the separation into three distinct categories in Table 7 is not quite accurate. Hypoxia might damage DNA in replicating cells and certainly irradiation can kill cells as well as damaging DNA. It is not possible to achieve a complete separation of agents into one type or another. With varying exposure levels there may be considerable overlap among the three types of cellular effects and the behavioral consequences. However, information is accumulating rapidly in the field of behavioral teratogenesis. It is important to begin to look for common behavioral consequences and to consider the common categories of causation into which fetotoxic agents may be placed.

TABLE 7. TENTATIVE CLASSIFICATION OF BEHAVIORAL TERATOGENESIS

CNS Effect	Immature Behavior	Adult Behavior
Cell Death	Hyperactivity	Recovery
Cell Genetic Damage	None	Hyperactivity
Cell Differentiation Delay	Maturational Delay	Recovery

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## SENSORY TESTING IN OCCUPATIONAL MEDICINE

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Sensory testing plays an important role in current neurological practice. This role is marked by the routine inclusion of many standard tests (such as the visual acuity chart and tuning fork) in the clinical examination (DeJong, 1979). However, such testing procedures have not as thoroughly permeated the field of occupational medicine. Furthermore, there is now increasing awareness that these simple tests alone may prove inadequate in detecting incipient toxicity, a major goal of occupational medicine.

Most clinical tests of sensory function are deficient in two ways. First, they usually sample a narrow range of functions within a sensory modality and therefore can easily overlook toxic effects on other aspects of sensation. For example, despite profound impairment of color vision (Raitta et al., 1978), victims of n-hexane poisoning may have normal visual acuity. Second, even when examining the correct function, clinical tests may only be adequate for detecting profound losses of sensation. The tuning fork, used to measure a patient's sensitivity to vibration, is such an example. More refined techniques for measuring vibration sensitivity are needed to detect and quantify even subtle alterations of somatosensory function.

This paper will examine the occupational toxicity of visual and somatosensory systems with the goal of selecting the most sensitive and informative clinical and laboratory tests. The relative value of various tests will be judged by their sensitivity to selective toxic effects on these two sensory systems. A similar approach can be taken to the study of toxic effects on audition, olfaction, and gustation, but this will not be discussed here.

### VISUAL SYSTEM

The range of chemicals found in occupational settings that are capable of damaging the visual system is enormous. A few examples are listed in Table 1. One can imagine that the task of detecting visual toxicity would be overwhelming if the signs of poisoning due to each of these agents were unique. Fortunately, however, the effects of many toxicants and drugs converge on



a limited set of visual dimensions. In the following section, we will point out some functions commonly affected by chemical insult, and describe techniques developed to test them. Thorough and multifaceted sensory testing is usually necessary for the detection and differential diagnosis of toxic disorders of vision. We will not consider visual toxicity of the cornea, lens or retina that can be directly observed by a physician with the aid of a slit lamp or ophthalmoscope (Grant, 1974).

TABLE 1. TOXICANTS AFFECTING VISION

ARSENICALS  
CARBON DISULFIDE  
n-HEXANE  
LEAD  
METHANOL  
TRIETHYL TIN

### CENTRAL AND PERIPHERAL VISION

The center of the visual field (center of gaze) is specially adapted for resolving fine details. The great importance of such details in our routine activities forces us to continually move our eyes to position interesting objects in this central region. Automobile drivers sequentially scan the important features of the environment with their central vision: oncoming cars, road signs, rear view mirror, speedometer, etc.; they use their peripheral vision to provide orienting and alerting information.

This distinction is important for our purposes because some chemicals selectively damage central vision while others destroy peripheral vision (Merigan, 1979). Central vision is the target of methanol, lead, carbon disulfide, and thallium, as well as other environmental chemicals and drugs (Grant, 1974; Potts, 1980). The pathologic basis of this effect may be a selective vulnerability of the papillo-macular bundle, a group of nerve fibers arising from the central portion of the retina (Potts and Gonasun, 1975). Sensory testing of central vision usually relies on the unique ability of this region to resolve fine details. Traditional visual acuity tests are most useful, although color vision and sensitivity to motion are also valuable assays of central vision (Merigan, 1979). Central deficits can also be detected by a perimetry technique (Aulhorn and Harms, 1972) in which small test stimuli are used to measure the sensitivity of discrete regions throughout the central visual field.

An equally diverse group of toxicants can impair peripheral vision including arsenicals, carbon monoxide, chloroquine, and quinine (Merigan, 1979). Pathologic studies indicate that these chemicals appear to damage ganglion cells or the pigment epithelium in the retina (Grant, 1974; Potts, 1980). Methylmercury impairs peripheral vision by damaging a portion of visual cortex (Takeuchi, 1962). Perhaps the most sensitive technique for detecting alterations in peripheral vision is the measurement of visual fields (Harrington, 1976; Merigan, 1980). This technique maps the most peripheral locations in the visual field at which a small test spot can be detected. It can be used to

identify even small and irregular losses of peripheral vision. The major drawback of visual field measures is the difficulty and expense of obtaining them because of the requirement for complex equipment, a skilled operator, and great patient cooperation. Given these practical obstacles, it would be most helpful if a simpler test were devised for peripheral vision which would be suitable for field testing of workers. Experiments in our laboratory (Merigan, 1980; Merigan, in preparation) indicate that temporal resolution (flicker detection) measured with large stimuli is a very specific test for peripheral vision. The critical feature of this test is the use of a stimulus which covers a wide region of the visual field. Such a target optimally stimulates peripheral vision because of the broad spatial integration found near the borders of the visual fields. In our studies of non-human primates we have found that reduced temporal resolution reliably accompanies visual field constriction.

### COLOR VISION

Many toxicants and drugs alter color vision (Grant, 1974; Verriest, 1972, 1978). It is not clear whether these effects reflect neural or photopigment alterations. A recent report of particular interest for occupational medicine (Raitta et al., 1978) described changes in color vision of workers exposed to n-hexane. While numerous techniques are available for assessing color vision (Verriest, 1978), the Farnsworth-Munsell 100 hue test (Farnsworth, 1943), which was used in the study of Raitta et al. (1978), is particularly useful in visual toxicity testing. The Farnsworth-Munsell test is easily administered, and can reveal both the nature and degree of color vision deficiency (Wright, 1972). Foulds et al. (1970) and Koliopoulos and Palimeris (1972) have also used this test to examine the toxic impairment of human color vision.

### OTHER ASPECTS OF VISUAL FUNCTIONS

There may well be additional dimensions of visual toxicity whose importance will become clear with further study. Fox and Sillman (1979) have suggested that lead and other heavy metals may damage rod (but not cone) photoreceptors. Techniques are available to test the separate contribution of rods and cones to visual function. In one test, rod function is examined by dark adapting the patient and measuring visual capacities with very dim targets. In a second method (Stiles, 1959), rod vision is tested while cone input is minimized by flooding the eye with intense red light. Conversely, cone vision may be tested while rod input is suppressed with intense blue adapting light.

A second example involves the demyelinating effects of such occupational and environmental toxicants as arsenicals, trialkyl tins, thallium, and tellurium. The visual consequences of demyelination in multiple sclerosis have been extensively studied (Regan et al., 1977), and may provide a model for examining the visual effects of the above agents. Tests of visual acuity have been useful in detecting both multiple sclerosis (Regan et al., 1977) and experimental demyelination of the optic tract (Jacobson et al., 1977). However, the sensitivity of these tests can be greatly increased if, in addition to visual acuity, the contrast sensitivity of the patient is determined (Regan et al., 1977). In this increasingly popular technique, spatial vision is

tested with grating patterns. These test patterns vary in bar width (or spatial frequency) from extremely broad bars to striations so narrow as to exceed resolution. For each pattern, the contrast between the light and dark bars is gradually reduced until the patient can no longer detect the pattern. Regan et al. (1977) have found that the resulting contrast thresholds are exquisitely sensitive to demyelination caused by multiple sclerosis. Often sensitivity to broad bar patterns was greatly reduced whereas visual acuity remained normal. The analogy to multiple sclerosis suggests that this test is well suited to the detection of subtle visual deficits in populations exposed to demyelinating agents.

### SOMATOSENSORY SYSTEM

Many physical and chemical agents found in industrial contexts have caused peripheral nerve disorders as reflected by cutaneous sensory dysfunction. Some of these compounds, such as n-hexane (Herskowitz et al., 1971) and methyl n-butyl ketone (Billmeyer et al., 1974; Mendell et al., 1974; Wickersham and Fredericks, 1976) are used as solvents. Carbon disulfide (Knave et al., 1974) and acrylamide (Garland and Patterson, 1967; Takahashi et al., 1971) are neurotoxic chemicals of importance in plastics and rubber manufacturing. Arsenic (Garb and Hine, 1977; Feldman et al., 1979) and thallium compounds (Gastel, 1978) are found in insecticidal sprays and dusts and in glass. Organic mercurials (Hunter et al., 1940; Ahlmark, 1948) serve as seed dressings, triorthocresyl phosphate as a plasticizer (Susser and Stein, 1957), and methyl bromide (Kantarjian and Shaheen, 1963) as a refrigerant and fumigant.

New studies keep extending the list of neurotoxic agents. For example, ethylene oxide, which is mainly known as a precursor for industrial chemicals, is also extensively employed in the sterilization of heat-sensitive hospital equipment. Several workers were recently exposed to this gas through a leak from a large ethylene oxide sterilizer, and shortly thereafter developed neurological sequelae partially involving the peripheral nervous system (Gross et al., 1979). In another factory, several workers experienced signs and symptoms of sensory neuropathy from formulating an ant pesticide, chlordecone, better known as Kepone (Martinez et al., 1978). More recently, introduction of a new catalyst in the polyurethane foam factory added dimethylaminopropionitrile to the list of chemicals able to induce sensory nerve disorders (Keogh et al., 1980; Kreiss et al., 1980).

Intoxication by any of these chemicals can result in sensory-motor dysfunctions of the extremities expressed in a stocking-and-glove distribution. Signs and symptoms include numbness, tingling, burning sensations, paresthesias, and dysesthesias as well as impaired cutaneous sensation. However, recent findings suggest a degree of specificity in both the pathologic and functional expression of peripheral neurotoxicity.

### EFFECTS ON LARGE MYELINATED FIBERS

The peripheral nervous system contains both myelinated and unmyelinated fibers. The fiber diameter histogram for myelinated fibers is bimodal. The peak of large myelinated fibers is found at approximately 10 microns diameter

and the peak of small fibers at around 4 microns. Unmyelinated fibers have axons of about 0.2 to 3.0 microns in diameter. These morphologic distinctions are important because they are associated with differential vulnerability to toxic insult.

Sural nerve biopsies from intoxicated humans have shown arsenic induced peripheral neuropathies and drastically reduced densities of large diameter myelinated fibers, whereas the population of small diameter fibers was fairly intact (LeQuésne and McLeod, 1977). Such a differential vulnerability of large fibers is also seen after intoxication with lead (Fullerton, 1966), triorthocresyl phosphate (Cavanagh, 1964), and acrylamide (Hopkins, 1970; Hopkins and Gilliatt, 1971; Sumner and Asbury, 1974).

These cutaneous myelinated fibers of large diameter primarily serve the sense of touch, vibration and two-point discrimination. Several techniques are available to test these sensations. In the conventional neurological examination, touch, two-point discrimination and vibration sensitivity are typically tested with a cotton wisp, a pair of calipers and a tuning fork, respectively. Although these screening tests are suitable for routine examination, they may be inadequate for detecting subtle deficits. Thus new techniques have been devised to provide quantitative measures of cutaneous sensitivity.

Electrodermal stimulation provides a convenient, easily calibrated measure. Electricity applied to the skin first excites low resistance cutaneous fibers. Since resistance of nerve fibers is inversely related to fiber diameter, low intensity electric stimulation offers a convenient way of testing the functional integrity of large diameter fibers (Uttal, 1957; Hahn, 1958; Rollman, 1969). The techniques of electrocutaneous stimulation are well developed and have been successfully applied to the study of neurologic diseases (Conomy and Barnes, 1976).

The functional role of large myelinated fibers can be examined in greater detail with tests of vibration sensitivity. Electrophysiological and psychophysical studies suggest that the detection of low and high frequency vibration is mediated through two different populations of receptors (Verrillo, 1963, 1965; Mountcastle, 1967, 1972). Pacinian corpuscles, on account of their viscoelastic properties, are the receptors of high frequency vibration. The receptors for low frequency vibration have not been identified anatomically, although Meissner corpuscles are likely candidates. Unfortunately, it has proven difficult to develop appropriate equipment for measuring vibration sensitivity. Most devices from the tuning fork and its electrified versions (Gray, 1932; Collens et al., 1946), to more elaborate vibrators (Laidlaw and Hamilton, 1937; Edwards et al., 1973; Arezzo and Schaumburg, 1980) have technical limitations. Recently, however, a computerized system for the quantitative study of vibration sensitivity (based on the work of Verrillo and Mountcastle) has been developed at the University of Rochester. An electromagnetic vibrator delivers stimuli whose amplitude and frequency can be independently controlled. This apparatus has been used to assess vibration sensitivity in humans and monkeys (Maurissen, 1979; Maurissen and Weiss, 1980), a measure which has proven sensitive to toxic damage of the peripheral nerve (Maurissen, in press).

Vibration sensitivity can also play a role in exploring specific toxic effects on cutaneous end-organs. Investigation of the dying-back neuropathy produced by acrylamide has shown that sensory nerve terminals degenerate before motor terminals. The hierarchy of sensory terminal vulnerability to this agent is headed by Pacinian corpuscles, which are the first to degenerate, followed by primary, and, later, secondary muscle spindle afferents (Schaumburg et al., 1974). Pathophysiological examination of Pacinian corpuscles discloses impaired mechanosensitivity at a time when no ultrastructural alterations can be detected (Spencer et al., 1977a,b). Triorthocresyl phosphate also induces ultrastructural changes in the Pacinian corpuscles, which may precede atrophy of the nerve terminal (Krishnamurti et al., 1972). Neurohistological and neurohistochemical studies with this compound have also revealed degeneration of peripheral sensory organs (Cavanagh, 1963; Vij and Kanagasuntheram, 1972).

The fate of Meissner corpuscles after intoxication has received less attention. Ridley (1968) reported a decreased Meissner count in a case of thalidomide neuropathy. Dickens and associates (1963) described a case of sensory neuropathy possibly due to organic solvent exposure where a dermal biopsy showed an absence of cholinesterase-reacting Meissner corpuscles. These studies suggest that somatosensory end-organs can be markedly damaged by exposure to toxic chemicals. Vibration sensitivity measures are a powerful technique for testing the functional integrity of these receptors.

#### UNMYELINATED AND SMALL MYELINATED FIBERS

Other toxic chemicals can selectively reduce the number of small diameter fibers and leave the large fiber population relatively untouched. Chlordecone, a chlorinated hydrocarbon insecticide, predominantly destroys unmyelinated fibers (Martinez et al., 1978). Clioquinol, an intestinal amebicide, is responsible for the epidemic of subacute myelo-optic neuropathy (SMON). SMON patients have been classified into sensory and sensorimotor types. Nerve biopsies from patients suffering from the sensory syndrome revealed that small myelinated fibers were affected more than large myelinated fibers (Tohgi et al., 1977). Another example of selective degeneration is that of capsaicin, the pungent constituent of red pepper. Administered to neonatal rats, capsaicin caused selective degeneration of unmyelinated fibers (Jancso et al., 1977).

Unmyelinated and small myelinated fibers mainly serve the sensations of pain and temperature. A classical distinction is made between the sensation of cold conveyed by small myelinated fibers and warmth conveyed by unmyelinated fibers (Darian-Smith, 1977). Some large cutaneous fibers, however, can also play a role in transmission of pain (Willer et al., 1978). A distinction is also made between first (or acute) and second (or constant) pain sensations, which are transmitted by small myelinated and unmyelinated fibers, respectively (Nathan, 1977).

In the clinical examination, pain and temperature sensitivity are usually assessed with a pin and test tubes containing hot or cold water. More elaborate equipment is available. For example, temperature sensitivity can be assessed with the "Minnesota thermal disks" (Dyck et al., 1974). They consist of four

disks made of materials which have different heat transfer characteristics. Placed on the skin, these disks have distinctive degrees of coldness. Advances in semiconductor technology have led to the development of new thermostimulators. They generate the thermal stimuli necessary to accurately measure cold, warm, and pain thresholds. Such devices have also been adapted to the study of sensation in patients suffering from nerve disorders (Fruhstorfer et al., 1976; Hamann et al., 1978). As evidence continues to accumulate of selective toxicities in the somatosensory system, these tests may become increasingly valuable for the detection and differential diagnosis and followup of peripheral neuropathies.

In summary, exposure to many industrial and environmental chemicals can cause impairment of visual and somatosensory function. Such effects can be detected at a very early stage with sensitive and carefully chosen sensory tests. In selecting these tests three characteristics are of primary importance; tests should be quantitative, sensitive to subtle alterations in sensory function, and they should examine those capacities most subject to toxic insult.

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#### ACKNOWLEDGEMENTS

This work was supported in part by Grant MH-11752 from the National Institute of Mental Health, by Grants ES-01885, ES-01248 from the National Institute of Environmental Health Sciences, and in part under Contract No. DA-AC02-76EVO3490 with the U. S. Department of Energy at the University of Rochester, Department of Radiation Biology and Biophysics, and has been assigned Report No. UR-3490-1936.

## HUMAN OCCUPATIONAL BEHAVIORAL TOXICOLOGY

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### INTRODUCTION

The passage of the Occupational Safety and Health Act brought into action a program intended to help identify and prevent worker's exposure to occupational safety and health hazards. Implementation of this law has served to help increase public awareness of the possible deleterious effects of toxic substances found at the workplace. One needs only consider such examples as worker's exposure to asbestos (carcinogen), Kepone (neurologic and reproductive effects), or dibromochloropropane (male sterility) to realize the serious hazards posed by some industrial exposures. Clearly, the best means by which to reduce such occurrences is through a program of prevention. A necessary component of a prevention program is to detect early the functional effects of a toxic substance prior to the development of irreversible, pathologic damage. With regard to the nervous system, one approach to early detection of functional effects is through use of behavioral procedures. The use of methods and procedures from the behavioral sciences to detect the effects of toxic substances is called Behavioral Toxicology.

The purpose of this paper is to provide an overview of one aspect of behavioral toxicology, occupational behavioral toxicology. The content of this paper will therefore be restricted to investigations of humans exposed to chemicals of occupational relevance. In addition, the emphasis will be on human workplace investigations.

As stated previously, this paper will present an overview of occupational behavioral toxicology. As the term is used in this paper, occupational behavioral toxicology refers to human laboratory and workplace investigations, and for which the concern is the effect of industrial chemicals on behavior. Also, it should be understood that "behavior" is used in its broadest sense, i.e., can include: a) human performance on structured performance tasks; b) subject's responses on measures of subjective distress scales (e.g., personality inventories), or; c) observations made by clinicians of abnormal behavior. The intent of this paper is to acquaint the reader with the following: types of occupational behavioral toxicology studies, problems and limitations of such studies, and future directions for occupational behavioral

toxicology. For the interested reader, a more comprehensive description of occupational behavioral toxicology can be found in another source (Johnson and Anger, 1981).

The premise underlying the initial development of behavioral toxicology as a science was that impairment of behavior would be among the earliest indicators of an organism's response to a toxic substance. Early detection meant preventive action could be taken prior to the development of a pathologic condition. To an extent, the premise has proved to be valid - though by no means has the "early detection" of toxic damage been the outcome of all behavioral investigations. Nor has occupational behavioral toxicology yet become the end-all in providing a sensitive tool for use in preventive medicine. Human behavior is too complex for such a simplistic concept. Still, occupational behavioral toxicology, within its limits, has much to be commended for the study of workplace toxic substances. Some important uses of occupational behavioral toxicology include the following:

- a) In appropriately designed studies, as an indicator of functional impairment of the nervous system at toxicant exposure levels below those which produce overt tissue or organ damage;
- b) as a noninvasive means for monitoring the effects of worker's exposure to toxic substances;
- c) as a complement to neurological methods as a means of providing information about the central nervous system;
- d) as an indicator of impaired behavior that could compromise a person's safe job performance;
- e) as an indicator of worker's exposure levels to toxic substances that may require development of occupational standards.

In summary, occupational behavioral toxicology, while still in the developmental stage as a science, can provide the clinician or toxicologist with evidence of the functional effects of toxic chemicals, particularly those whose target is the nervous system.

#### HISTORICAL DEVELOPMENT

To appreciate the current status of behavioral toxicology, a historical review of this science will help provide a greater perspective.

The beginnings of what is now called behavioral toxicology occurred in the United States in the early 1960's. The initial interest in behavioral toxicology sprang from three sources. First, the study of the effects of drugs on behavior (behavioral pharmacology) had become relatively well accepted, and some behavioral investigators (Armstrong et al., 1963) saw the opportunity to use similar methods for the study of toxic industrial substances. Second, batteries of neuropsychological tests had been designed to evaluate organic brain damage and developmental behavioral disorders. A third, and

ultimately more important, factor in shaping the interest in behavioral toxicology was the need for government regulation of toxic substances in the community environment and at the industrial workplace. The public concern against environmental pollution grew, and this concern prompted Congress to create the mechanisms by which the Government would control potential exposures to environmental pollutants and toxic substances. Legislation such as the Clean Air Act, the Occupational Safety and Health Act, and the Toxic Substances Control Act provided the necessary legal basis for government agencies to investigate and establish exposure limits for toxic chemicals, plus the financial support to fund research efforts dealing with such problems.

Aided by the new interest of regulatory and standards settings groups, behavioral toxicology gradually began to grow. Work by Goldberg et al. (1962) on the behavioral effects of industrial solvents marked an early beginning. Later, Beard and Wertheim (1967) reported that human exposure to low concentrations of carbon monoxide impaired subject's performance on a laboratory task requiring auditory time discrimination. This report ultimately had a major impact on the establishment of a Federal air quality standard for carbon monoxide. The Beard and Wertheim study, because of its implications for regulatory actions, played a major role in the development of interest in behavioral toxicology in the United States and further stimulated funding of behavioral toxicology research by government agencies.

During the 1970's interest grew in behavioral toxicology along three fronts: 1) investigations of effects of toxic substances on operant behavior in laboratory animals; 2) human laboratory studies of effects of low concentrations of workplace chemicals on structured human performance tasks, and; 3) cross-sectional epidemiological and clinical studies of workers occupationally exposed to substances thought to adversely affect the nervous system and human behavior.

Of late, behavioral toxicology has progressed beyond simply obtaining information that may be useful in standards setting and regulatory action. Such information has been typically developed in studies of groups and, as such, is not easily applied to individuals comprising the group. The direction is now to develop additional behavioral tests that are predictive of early signs of toxicity, but which also permit interpretation for individuals, not just groups. The development of such tests will require standardized methods and norms for populations that are adjusted for sex, age, education and socioeconomic factors. While the task is formidable, progress toward that end is evident (Valciukas and Lilis, 1980).

#### TYPES OF STUDIES

The types of occupational behavioral toxicology studies have been grouped (Hanninen, 1981) according to the type of toxic effect expected, i.e., acute or chronic. Hanninen's classification is as follows:

1. Assessment of acute or subchronic effects
  - a) in experimentally induced exposures performed under laboratory conditions;
  - b) in workplace studies of occupationally exposed workers.
2. Assessment of the effects of long-term exposures
  - a) in prospective investigations of workers;
  - b) in cross-sectional analysis of relationships between worker's exposure levels and behavioral impairments, without the use of comparison groups;
  - c) in cross-sectional comparisons between groups of workers and a properly chosen reference group.

The type of occupational behavioral toxicology investigation chosen for implementation in a given situation will depend, of course, on the purpose of the study, the anticipated mechanism of toxicity of the toxic substances, and other factors. Illustrations of most of the aforementioned types of studies will be presented in a later section of this paper.

#### BEHAVIORAL METHODS

The specific behavioral methods utilized in a given investigation will be determined in large measure by the type of investigation being conducted. For example, human laboratory studies may be designed to examine in detail the effect of a toxic substance on one specific type of human behavior, e.g., performance on structured memory tests. Other types of investigations may choose to evaluate a broad range of behaviors, and as a consequence, a battery of behavioral tests would be administered.

Typical of the types of behavioral tests administered in occupational behavioral toxicology studies performed at the workplace are those found in the test battery developed at the Finnish Institute of Occupational Health (Hanninen and Lindstrom, 1976). The elements of this battery of tests are shown in Table 1. This particular battery has been used in more occupational behavioral toxicology investigations than any other. The functions being measured (e.g., memory/retention) are also shown in Table 1. A complete description of each test is beyond the scope of this paper, and the reader is referred to other sources (Hanninen and Lindstrom, 1976). Some brief comments, however, are in order. First, extensive use of tests from the Wechsler Adult Intelligence Scale (Wechsler, 1958) has occurred in occupational behavioral toxicology studies. These tests are attractive to the behavioral toxicologist for several reasons: 1) they possess normative data, which can sometimes be used for comparative purposes; 2) they are relatively easy to administer and do not require large amounts of time, and; 3) instrumentation requirements and scoring of results are not difficulties. As a general rule, these three considerations are desirable features for any behavioral test battery applied to groups of workers.

TABLE 1. ELEMENTS OF INSTITUTE OF FINNISH  
OCCUPATIONAL HEALTH BEHAVIORAL TEST BATTERY  
(HANNINEN AND LINDSTROM, 1976)

A. Wechsler Adult Intelligence Scale	B. Memory/Retention	C. Vigilance/Cognitive
1. Similarities	1. Wechsler Memory Scale	1. Bourdon-Wiersma Vigilance Test
2. Vocabulary	a. Logical Memory	2. Symmetry Drawing
3. Digit Span	b. Visual Association	3. Figure Identification
4. Digit Symbol	c. Associative Learning	
5. Picture Completion	2. Benton Visual Retention Test	
6. Block Design	3. Kuhnburg Figure Matching Test	
D. Coordination/Dexterity	E. Personality Variables	F. Self-Reported Symptoms
1. Santa Ana Dexterity	1. Rorschach Inkblot Test	
2. Finger Tapping	2. Eysenck Personality Inventory-C	
3. Mira Test		

### SUBSTANCES STUDIED

A recent review (Johnson and Anger, 1981) of the literature pertaining to occupational behavioral toxicology identified approximately 30 reports describing workplace investigations. There are also approximately 30 reports on human laboratory studies of occupationally relevant chemicals.

Table 2 contains a list of industrial chemicals that have been studied for behavioral effects (Johnson and Anger, 1981). The type of behavioral study, human laboratory (L) or workplace (W), is also noted in Table 2. A review of Table 2 shows that about 20 chemicals have been the subject of occupational behavioral studies. Of this number, studies of the behavioral effects of industrial solvents have predominated. The reasons for this predominance are twofold: a) the widespread presence of solvents in industry, and; b) demonstrated toxicity on the central nervous system of these compounds at high exposure levels.

Shown also in Table 2 are those chemicals for which behavioral studies have influenced exposure limit recommendations found in NIOSH criteria documents. As indicated, results from occupational behavioral toxicology studies have influenced the recommendations found in several criteria documents.



TABLE 2. CHEMICALS THAT HAVE BEEN INVESTIGATED IN  
OCCUPATIONAL BEHAVIORAL TOXICOLOGY STUDIES

<u>SOLVENTS</u>	<u>METALS</u>	<u>PESTICIDES</u>	<u>OTHER</u>
Styrene (W)*	Lead (W)*	Organophosphates (W)	Carbon Monoxide (L, W)*
Carbon Disulfide (W)*	Mercury (W)*	Leptophos (W)	Anesthetic Gases (L)*
Methyl Chloride (L, W)			Polybrominated Biphenyls (W)
Xylene (L)			Jet Fuel (W)
Methyl Ethyl Ketone (L)			Formaldehyde (W)
Paint Sprays (W)			
Methylene Chloride (L)*			
Trichlorethylene (L)*			
Perchloroethylene (L)			
Toluene (L)			

L = Human Laboratory Study

W = Workplace Study

\* = Permissible Exposure Limit (PEL) Impact

### ILLUSTRATIVE STUDIES

It is beyond the scope of this paper to provide a review of the literature on occupational behavioral toxicology. Such a review is available elsewhere (Johnson and Anger, 1981). However, in order to illustrate the types of occupational behavioral toxicology studies that have been conducted, the following reviews are provided of investigations performed by NIOSH. The first study, a workplace carbon monoxide investigation, is an example of a workplace study for which the acute effects of a toxic substance were of concern to the investigators.

#### CARBON MONOXIDE (Workplace Investigation, Repeated Measures Design)

A review of the literature revealed only one workplace behavioral investigation of workers for which the investigator's principal concern was CO exposure. Johnson et al. (1974) studied the effects of vehicle emissions on the behavioral performance of toll collectors stationed at a busy interstate highway. Strictly speaking, the workers were not exclusively exposed to CO, but to other constituents of vehicle exhaust (e.g., lead, ozone) as well. The investigation, though, concluded that the major pollutant was CO. The group selected for study consisted of six workers employed as fare collectors at a toll highway in central Kentucky. The study group consisted of five females and one male. The subjects ranged in age from 19 to 49 years. All subjects were nonsmokers in order to eliminate this source of CO. The six workers were tested, three per day, on alternating days over a twelve day period.

Preceding the start, tests were administered prior to each worker's workshift and following completion of the workshift. Testing was conducted in motel rooms adjacent to the toll plaza where ambient CO levels were always less than 5 ppm. The performance tests consisted of the following: a) both simple and choice visual reaction time; b) hand-eye coordination, using the Michigan test; c) visual function (critical flicker frequency, pattern identification presented via a tachistoscope); d) an arithmetic test, and; e) a dual task. The experimenters controlled the difficulty of the dual task, and as its difficulty increased, the effect was to reduce subject's performance.

In addition to the behavioral tests, biological data (breath CO and COHb) and workplace industrial hygiene data (air lead, ozone, and noise) were also obtained. The investigators found that when preshift vs. postshift performance changes were correlated with increases in COHb due to workplace exposure, there were statistically significant ( $p < .05$ ) associations between impaired dual-task performance as COHb increased. No associations between COHb and impaired performance on the other behavioral tests were found.

#### CARBON MONOXIDE (Laboratory Investigation)

The results from this study of toll collectors led to laboratory investigations of the acute toxic effect of CO on time-shared human performance. The experiment described below illustrates the utility of a laboratory study as a means of clarifying findings from workplace exposures. Given the limitations of the field investigation, e.g., only six subjects, how valid was the finding that CO would affect complex human performance under conditions where the subject's reserve performance capacity was decreased? To answer this question, Putz and colleagues (1976, 1979) designed a series of laboratory experiments that investigated the effects of CO on task time-sharing and vigilance. In two separate studies, using different experimental designs but identical performance tests, Putz et al. found that COHb levels as low as 5 percent degraded subject's performance on a time-shared task when the task performance was difficult. The degraded performance took the form of: a) increased time to respond to detect signals presented in the peripheral visual field, and; b) reduced accuracy on the psychomotor tracking task used concurrently with the peripheral field signal detection task.

The findings from the NIOSH studies on carbon monoxide show that acute CO exposures, at levels sufficient to yield 5 percent COHb, will degrade complex, demanding performance in humans unacclimatized to CO. The salient feature is that the subject's reserve capacity must be diminished by the task, that is to say, the performance must be difficult. What this suggests is that low level CO exposure will not affect worker's safe performance of routine, non-demanding jobs; however, it may affect performance of jobs requiring rapid response to complex situations.

### LEPTOPHOS (Cross-Sectional, No Comparison Group)

In 1977 NIOSH was advised of 12 cases of serious neurological disease that had occurred in workers at a plant manufacturing Leptophos, a potent pesticide. Leptophos, though not approved for use in the U. S., was manufactured for sale in non-U. S. markets. Early in 1977, 155 workers (both current and former) were given comprehensive medical examinations that evaluated general medical status, neurological status, and measures of neuromuscular, ophthalmologic, psychological, and biochemical function (Xintaras et al., 1978). The following performance tests (and function tested) were administered to each participant: Santa Ana dexterity (hand-eye coordination), choice reaction time, Neisser Letter Search (visual organization), and three subtests from the Wechsler Adult Intelligence Scale (WAIS): Block Design (perceptual organization), Digit Symbol (memory efficiency), and Digit Span (recent memory). A complete occupational history was obtained for each worker. A comparison group was not included in this study, but rather, laboratory and literature norms were used for the various tests. Therefore, this study is an example of a cross-sectional behavioral study, but without the inclusion of a comparison group. Additionally, exposure and effect relationships were explored statistically within the study group. Results showed statistically significant ( $p < .05$ ) performance decrements in Block Design, Choice Retention Time, and Santa Ana Dexterity. Since all three tests are strongly associated with peripheral nerve function, the investigators felt the performance decrements were suggestive of peripheral nerve dysfunction. There was some evidence, though not complete, from the neurological exams to support this hypothesis. A confounding factor in the interpretation of the results was the discovery that many workers had also been exposed to n-hexane, a potent neurotoxic solvent. The investigators were consequently unable to determine the causative agent in inducing worker's performance decrements. In this regard, the behavioral results were important in a clinical sense, since functional impairments in workers were identified. However, as a toxicology study, the association between cause and effect could not be established.

### LEAD (Cross-Sectional, with Comparison Group)

As an example of a cross-section study, with use of comparison groups, Johnson et al. (1980) evaluated neurobehavioral functions and personality variables in 403 workers at a primary nonferrous smelter. Community residents not having a history of lead exposure served for comparison. Study groups of smelter workers were formed according to job title. Neurobehavioral tests consisted of visual choice retention time and hand-eye coordination. Personality variables were assessed using the Multiple Adjective Affect Checklist. Current PbB level and ZPP concentration were obtained for each study participant, as were occupational history, neurologic measures, and self-reported symptoms. The highest group mean

PbB was found to be 56 µg/dl for male workers in the lead smelter. Analysis showed the smelter workers were about 10 percent slower in reaction time, but did not differ in hand-eye coordination. Using clinical norms, smelter workers were more depressed and hostile than unexposed workers.

### FUTURE DIRECTIONS

It seems clear that occupational behavioral toxicology has contributed to a more complete understanding of the effects on workers of industrial hazards. It is also apparent that occupational behavioral toxicology, to be more useful, will require additional development. The nature of some of these future developments is the subject of this section.

### SENSITIVITY

The ability of behavioral tests to detect, in a reproducible manner, small changes in central nervous system functions needs to be improved. Current test methods and procedures have been derived in large measure from standard tests, such as the Minnesota Multiphasic Personality Inventory and the Wechsler Adult Intelligence Scale, designed for more general purpose applications. There exists a need for development, and validation, of psychological tests that can be administered to worker populations exposed to low amounts of toxic substances. Of particular importance is the development of valid tests to evaluate the effects of toxic substances on: a) cognitive functions, and; b) personality factors. Presently used tests, such as the WAIS and the Eysenck Personality Inventory, were developed for clinical applications and, as such, do not possess the sensitivity to detect subtle changes in personality or cognitive functions.

The evaluation of sensory and psychomotor functions is more advanced than are tests of personality and cognitive functions. This results in large measure from the large body of literature on psychometrics. There is, however, a need to develop better normative data for the sensory and motor functions tests currently in use.

### SPECIFICITY

The previously described NIOSH investigation of Leptophos (Xintaras et al., 1978) illustrates one limitation of occupational behavioral toxicology studies, namely, the lack of specificity of the behavioral tests employed in that study. Results from the aforementioned study showed functional impairment of the workers, but the investigators could not associate this impairment with a specific toxic agent. This lack of specificity is a problem common to many workplace investigations. Simply put, a number of toxic substances can yield the same pattern of behavioral impairment, using the behavioral tests currently employed in most worksite studies. From a toxicologic standpoint, this lack of specificity is unacceptable. What is clearly indicated is the need to develop behavioral tests that possess diagnostic and predictive specificity. The development of such tests would proceed primarily from laboratory studies, but would then require field evaluation and validation.

## EFFICIENCY

In addition to improvements in sensitivity and specificity, there exists a need to improve the efficiency of behavioral tests used for workplace studies. As used here, efficiency refers to the time required for administration of the tests to each study participant, plus the experimenter's time to analyze the results. Presently used behavioral test batteries are inefficient in the sense that each subject's total testing time may approach 2 to 4 hours. While this amount of time, or longer, may be acceptable for many laboratory investigations, it often presents a problem for workplace studies, especially those for which workers are being given time off the job for the purpose of biomedical evaluation. The need therefore exists for the development of behavioral test batteries that tap a range of behaviors, but do not unduly impose a time-to-test burden on each participant.

## SUMMARY

Occupational behavioral toxicology has contributed to our knowledge of the functional effects of a substantial number of industrial chemicals. This knowledge has been important in several instances in establishing permissible exposure limits for the workplace. Additionally, identification of impaired behavior has served as an early warning indicator of toxicity which, if left unaltered, may result in irreversible pathologic damage. There remains, however, a need to improve the sensitivity, specificity, and efficiency of behavioral test batteries currently used in workplace investigations.

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#### ACKNOWLEDGEMENT

The author is pleased to acknowledge the contributions of his colleagues, Drs. Kent Anger, Vernon Putz-Anderson, Charles Xintaras, and Mr. James Setzer, in the performance of the NIOSH investigations described in this paper. The typing of the manuscript by Mrs. Mary Swenk is gratefully appreciated.

## OPEN FORUM

MR. VERNOT (University of California, Irvine): I have a question for Dr. Reiter. In your estimate of the behavioral toxicity index you used the LD<sub>50</sub> of the particular toxic material as the numerator. In your experiments with the carbamates, did you measure any type of cholinesterase activity as a possible alternative to the LD<sub>50</sub> in designing a toxicity index?

DR. REITER (U. S. Environmental Protection Agency): Since those original experiments were done, we've conducted further studies and have treated animals with the ED<sub>50</sub>s of both Baygon and Carbaryl and then measured five minutes of activity in a figure eight maze. Immediately after that behavioral testing, the animals were sacrificed and both blood and brain cholinesterase levels were determined. This was done in order to get a time course for the peak inhibition of cholinesterase and to relate the time pattern with other behavioral effects that were seen. The earliest we sacrificed the animals was 15 minutes post-injection which was the time the behavioral testing was done in these series of experiments and also corresponded with peak cholinesterase inhibition. The cholinesterase inhibition was about 55% in both blood and brain tissues. We did see a differential effect on recovery of cholinesterase activity between Baygon and Carbaryl. We thought that the onset of cholinesterase inhibition with Baygon would be much more rapid than with Carbaryl but we were unable to demonstrate that. At 15 minutes post-injection both compounds showed comparable cholinesterase inhibition, but the recovery after Carbaryl treatment is much slower than with Baygon and actually, activity depression returned to normal before cholinesterase levels.

MR. BOGO (Armed Forces Radiobiology Research Institute): Dr. Norton, I've seen reports that indicated it may be necessary to give a higher dose of radiation to a rat than a human to get a comparable type of effect. Was this true in your studies?

DR. NORTON (University of Kansas Medical Center): I don't think I mentioned the precise radiation dose we've been working with. We've used doses as low as 50R but most of our studies have been done at a dosing of 125R using a single exposure at a rate slightly under 40R per minute to the whole body of the mother. This is a rather high dose in terms of human exposures under ordinary circumstances or even for some mild accidents. It is not a high dose relative to nuclear war such as some of the exposures at Hiroshima and Nagasaki. There is plenty of evidence that when you do get up in the high dose range in humans you get into problems with the fetus which are not unlike the ones we're describing here. I don't think there's any dramatic difference in sensitivity in the dividing cells of the rat which is really what we're hitting. I think we're hitting the cells in the synthetic phase of DNA and I don't think there is a unique difference in the sensitivity of the neuroblast that's dividing at this time versus other dividing cells. Evidence that's been presented by some other people has proposed that it's possible with this kind of morphologic analysis to detect changes at radiation doses as low as 20 or 40R which is about minimum level for producing DNA damage. At that point we are getting down to the dose level to animals that may match a fair number of human exposures. Ionizing radiation is like many other toxic substances in producing dose related effects.

DR. HENDERSON (Olin Corporation): Is there any correlation of flicker fusion with blood or hair mercury levels in humans after methylmercury exposure?

DR. MERIGAN (University of Rochester): The technique I mentioned for measuring flicker fusion has not been used previously and I think the reason it has not is that people haven't analyzed how to test peripheral field function with that technique. The critical feature of the technique is that the stimulus has to be extremely large and if you use a very, very small flickering target you test primarily central vision as was done in testing methylmercury poisoning cases. There hasn't been any attempt to measure correlation between flicker fusion and these other indices you've talked about, although, of course, visual field measures do correlate with the blood and hair mercury measurements.

DR. HENDERSON: Dr. Johnson, as I recall, you said that at a 5% carboxyhemoglobin level you did see an impairment on timesharing. As I recall, 5% carboxyhemoglobin is what you will find in an average cigarette smoker. Is this correct? Do you see impairment after smoking one cigarette?

DR. JOHNSON (NIOSH): We've never studied cigarette smokers; in fact, we have specifically excluded smokers from our laboratory studies and the one worksite study that we did. Your original statement was correct. We did demonstrate impairment at 5% COHb, but it was in a situation that required sustained attention on the part of the subjects, the task was demanding and there were indications that the subjects perceived that task as being demanding. We felt that one of the principal findings of the study was to demonstrate the interaction between the complexity of the task and the biological effect. As far as relating COHb to what one might find in cigarette smokers, 5% is less than what you would find in many cigarette smokers. Heavy smokers will go as much as 8 to 10% COHb.

DR. REITER (U. S. Environmental Protection Agency): I'd like to follow up on the question. The data that you have is essentially from a vigilance task. How would you compare your results with the findings reported by Beard and Wertheim on the effects of carbon monoxide exposure?

DR. JOHNSON: The study reported by Beard and Wertheim has been a well discussed study, to say the least. One of the better discussions of that study, I think, is found in the review article by yourself and Dr. Laties. The task, as administered by Beard and Wertheim in the early 60's, was an auditory discrimination task in which the subjects were asked to discriminate the length or duration of one tone from another. The peculiar feature in that particular study was that the task was presented to the subjects in a very, very small confined exposure booth. The subjects may have experienced sufficient sensory deprivation to have caused the type of effect that was ultimately reported by Beard and Wertheim. Other investigators who have attempted to replicate that study have been quite unsuccessful. That does not mean, in my view, that the original study was flawed, but one needs to understand the context in which the study was conducted. As far as our study is concerned, we tried to vary the complexity of the task which was



not done by Beard and Wertheim. We also tried to minimize the potential problem of social and sensory deprivation that the subjects may have experienced in the Beard and Wertheim study. So there were a number of differences between the two. Basically, they are two different studies.

DR. KURTZ (Battelle Columbus Laboratories): This is for Dr. Zenick. One of the criticisms that's been offered for behavioral testing techniques has been that the interpretation of the results is questionable. Does not one exacerbate this problem when one uses a pharmacological tool, as you call them, that may have a multitude of pharmacological and behavioral effects?

DR. ZENICK (University of Cincinnati Medical Center): I have to give you somewhat of an ambivalent answer of both yes and no. For example, amphetamine is not the simplest compound to work with if you want to jump from looking at amphetamine effects to pinpointing neurochemical mechanisms. It's got a multitude of actions on the putative neurotransmitter system. It also is true that the effect you see with the probe doesn't necessarily have to be a CNS effect. It could be a reflection of liver metabolism. The initial concept of using the probe was not to get a better grasp of mechanism; rather, to disclose the deficit in a situation where one wasn't seeing any shift in baseline. I think, to clear up that problem one would need to use more specific probes with better delineated mechanisms of action and in the work we're doing now, we're trying other chemical probes. When we establish a drug discrimination paradigm, and begin to use blockers and agonists and antagonists, we like to work with compounds where those actions are clearly known. I think if your primary concern is the disclosure of a deficit, it probably is a reasonable approach to utilize. I don't believe that just that step alone gets you any closer to understanding mechanism.

DR. KURTZ: There have been those who have offered the interpretation, for example, that on the basis of the results that amphetamine brings out an effect due to lead exposure, there is an effect on the biogenic amine system. Is that a legitimate conclusion in your view or is it not justified?

DR. ZENICK: We're pursuing that point also. With respect to the lead question, if you name the neurotransmitter system and if you tell me what effect it is you want to see, I'll find you a paper that will show it. Our laboratory has traditionally been a neurotransmitter/neurochemistry lab and Dr. Michaelson has, in fact, published from that laboratory data showing alterations of biogenic amine systems. We're now backtracking a little bit in our research plans because one of the questions we're currently posing in our laboratory is that perhaps when you do study a lead exposure following a drug challenge, you may be seeing a perfectly normal response if you consider how much drug was concentrated in that area to begin with. What we're trying to do now is back off from a biogenic amine hypothesis and deal currently with the question of are they even taking the drug up and metabolizing it in the same manner. And if the answer to that is no, then that's one problem. The other aspect is that we are not dealing with the drug in the same way and the response the animals give to a certain amount of drug is abnormal as well. So we're trying to separate those

effects out. I don't think that steady state studies are going to help in understanding the neurochemical deficit associated with lead exposure.

DR. REITER (U. S. Environmental Protection Agency): I'd like to just add something to that, Dr. Zenick. I think the argument can be made from the other point of view and that is that one would strive to use pharmacological probes which were as non-specific as possible because the question that you're asking with those probes is about an impaired functional nerve in the brain and in that sense, you're on a fishing expedition. If you select pharmacological aids that are non-specific and which may have more than one mechanism, then you increase the likelihood if something's amok in the brain, that you would reveal that problem using these pharmacological probes. That then gives you a starting point at which you can now start to focus in on what the specific neurochemical problems associated with that difference is.

CAPT. REED (Air Force Rocket Propulsion Laboratory): Dr. Norton, you stated that the dose of X-irradiation used in your studies was 125R at 40R per minute. What energy or energies were involved in that dosage?

DR. NORTON (University of Kansas Medical Center): Since I'm not a biophysicist, I don't really feel prepared to answer that question. I can tell you what the exposure parameters were; the X-ray machine used a quarter of a millimeter of copper and one millimeter of aluminum. The distance from the source to the uterus was 50 centimeters.

MR. VERNOT (University of California, Irvine): A question for Dr. Norton. Did you determine what the level of carboxyhemoglobin was in the pregnant rats you made hypoxic?

DR. NORTON (University of Kansas Medical Center): Yes, we attained about 45% COHb in the highest level exposure. I think in the animals that I showed you, we used a 90-minute exposure to 4000 ppm in a dynamic flow chamber which produced a steady state of about 45% carboxyhemoglobin in approximately 60 minutes. That's a high dose of CO and the animals do lose consciousness.

MR. VERNOT: The animals lose consciousness at a 45% carboxyhemoglobin level?

DR. NORTON: Yes, they will respond to toe pinch or something like that, but left to themselves, they're out on their sides and they're not moving around at that point.

MR. VERNOT: Our experience is that rats, or at least the strains that we have used, are very tolerant to CO hypoxia and hypoxia of any kind.

DR. NORTON: We don't have any maternal death. This is not a dose which causes any subsequent damage to the mother as near as we can tell. Since the halftime of COHb is fairly long in blood, we take them out of the exposure chamber at 90 minutes and they still have high levels of COHb for about another hour. We can't prove this at this point, but we don't think that the effects we are getting are related to carbon monoxide except as it

produces anoxia in the fetus. When you have a fetus in a maternal situation where the mother has hypoxia, the fetus is more severely hypoxic. That's independent of the fact that to some extent the fetus is tolerant of hypoxia but not to the extent that they're really not at risk from hypoxia. Furthermore, fetal hemoglobin and its combining power with CO is essentially the same as normal adult hemoglobin.

DR. DUNGWORTH (University of California, Davis): Several of the speakers this morning commented on the batteries of tests they individually had used for looking at behavioral decrements. What I would like the members of the panel to comment on is how they would compare the sensitivity of the tests they used relative to the tests that the other speakers were talking about.

DR. MAURISSEN (University of Rochester): It's difficult to compare the sensitivity of these different tests in terms of vibration sensitivity and some of the sensory sensitivity. I've been interested in different compounds than those the other speakers talked about. I think somatic sensitivity is an interesting sensory medium to start with if you are talking about chemicals such as acrylamide that typically cause some peripheral numbness, tingling and paresthesia. Those are some of the early symptoms in cases of methylmercury poisoning. It's not possible to compare vibration sensitivity with a figure 8 maze in completely unrelated species like rats. There is no way to compare species while testing completely different functions. Depending on the chemical you are interested in, you would choose one or the other.

DR. MERIGAN (University of Rochester): Some of the tests that I mentioned of visual function which we do in human patients and in Macaque monkeys turned out to be extremely sensitive. The measurements made with gratings looking at a special vision contrast threshold is a very early change in many types of poisoning. We have chosen the monkey because of its great similarity in both structure and function in the visual system to humans and, in fact, we could not make many of our specific measurements in rats. For example, color vision is extremely different in any organism other than the monkey.

DR. REITER (U. S. Environmental Protection Agency): I think it's important when issues of sensitivity are discussed that one backs up and tries to determine what the question is. I think that the speakers this morning were addressing issues of behavioral toxicology at a multitude of levels of investigation. Let me see if I can put things in perspective by using the Toxic Substances Control Act (TSCA) as an example. You know, there are two sections of the TSCA; one deals with controlling new chemicals that are being introduced into commerce and the other section deals with compounds which are currently in commerce. If one is interested in toxicity of chemicals that are being introduced, you're asking in many instances quite different questions about that compound than if it's a substance which is currently in use such as lead or methylmercury where the target organs are fairly well identified. The question that you're asking is about what levels of exposure are producing these harmful effects. In the former case, you're asking is the compound toxic, what target organs is the compound toxic to, and is the

toxic hazard such that we should prevent it from being introduced into commerce. The testing procedures that I was addressing this morning were really aimed at that first question. When a compound of unknown toxicity shows up does it exhibit any CNS toxicity? That's the sole question. Dr. Merigan and Dr. Johnson's level of inquiry is at a considerably higher level than that. They are generally dealing with compounds where clinical observations would suggest that sensory systems are modified. They are not looking at these compounds as unknown materials. Again, I think the question of developmental neurotoxicology or neurotoxicity is one in which you're just asking a slightly different question. Is the developing organism of the developing nervous system more or less sensitive than the mature nervous system to these particular chemicals? The questions you're asking are quite different and to some extent, those questions are going to affect your choice of methods. Behavioral psychologists do a lot of in-fighting about relative sensitivity and I would argue with my colleagues that at a primary screening level certainly the figure 8 maze seems to have a fairly good track record. It's very easy to operate and I can generate a dose response curve for a chemical in one afternoon with the figure 8 mazes whereas it takes about two to three months to generate a similar dose response curve using operant techniques. However, the information that one derives from operant techniques is more extensive than what you get out of a simple measure of activity and there are other kinds of qualitative data that can be gathered that help to define what the behavioral disruption is. The fact that the figure 8 maze seems to have a fairly good sensitivity as a primary screening technique would not be a basis for eliminating operant techniques from behavioral toxicology because they have a place at a secondary level of testing or when one is asking specific questions about the toxicity of a particular compound.

DR. NORTON (University of Kansas Medical Center): I think I would like to answer the question in a slightly different way from the standpoint of behavioral developmental studies. There really isn't much difference in the test batteries which are used by people studying early development among different species of mammals because a neonate doesn't do very much unless you talk about precocious versus altricial animals where you're dealing with the state of brain development at the time of parturition. Guinea pigs are born a little bit more mature than some other species. There are those who work with the animals that are born in an altricial or helpless state where the brain is not fully developed which includes the rat, man, monkey and a large number of the other standard animals. The tests which are done in the early stages of neonatal development are fairly well defined simply by what you can get an animal to do at an early age. There you set up a battery of just about everything you can think of doing and make some decision as to what's likely to cause a problem in early age. For example, Dr. Voorhies and his co-workers have worked up a fairly extensive test battery which contains many of the tests that I've used. They did an extensive study on the effects of hydroxyurea, another compound like ionizing radiation that affects DNA. They concluded that they didn't get much effect on early developmental behavior at doses of hydroxyurea that should produce severe damage to dividing cells. They got the same kinds of results that we're getting with ionizing radiation. In developmental behavior studies, the limits as to what tests are chosen are really the limits of what a very immature animal can do.

DR. JOHNSON (National Institute for Occupational Safety and Health): The type of work I reported on is somewhat different from those of the other speakers, so as a consequence my answer is going to be somewhat different. The approach that I described this morning in terms of using a general battery of tests that examine behavioral functions along several lines is the most common approach. There aren't many people doing field investigations of behavioral activity but those who are all seem to have approached the problem from the same direction and use a battery of tests as described this morning. Problems that one encounters at the work site in terms of administering such tests, shape the types of tests that you take into the field. One has to be aware of the learning requirement of tests. If you're going to conduct a cross-sectional study and you don't have access to the workers for an unlimited amount of time, you can't use tests that require a great amount of learning. That restriction shapes the type of tests you can use in the field. You also need some normative data if at all possible to take into a test situation. That doesn't rule out new tests that might be developed in a laboratory and then applied in a field situation, but I think you need to have some normative data for those tests before you apply them in a field situation. You don't generally have a great amount of space provided to you in a field situation and you have to select your equipment accordingly. The time to administer the tests is important and in some cases has become a matter of negotiation between NIOSH and some companies we have studied. Lastly, the types of tests used are also influenced by the degree to which clinical data may be available about the validity of the tests. So in a general sense, those are considerations that guide the selection of tests that can be used in a field situation. The only direct comparison I can make between the tests that I have used for measurement of sensory function and those described by other speakers today would be a comparison of our tests of visual function with those reported by Dr. Merigan. For the reasons I have just stated in terms of time to administer and minimum learning requirements, the visual function tests we have used are far less sensitive than those he has described.

DR. ZENICK (University of Cincinnati Medical Center): We are working at developing more refined tests to assess specific behavior response when the general test tends to suggest some type of deficit. There are a number of programs conducting inter-laboratory comparisons of tasks where the laboratories take the same drugs or compounds, use the same battery of tasks and compare results. In this program it isn't important which task shows the most significant effect, but what is the inter-laboratory reliability of that determination.

DR. REITER (U. S. Environmental Protection Agency): The approach using within and between laboratory comparisons always seems to put people working in this area on the defensive and I think maybe that's appropriate. There was an inter-laboratory study done in Europe where they compared LD<sub>50</sub> determinations on the same chemicals and the variability that was obtained in that study was horrendous. If you search the literature for LD<sub>50</sub> values on compounds like chlordimeform and carbaryl, you find values that differ by as much as five-fold which illustrates that there are many variables that toxicologists don't seem to have good control of and it's not just limited to the area of behavioral toxicology.

DR. HENDERSON (Olin Corporation): Dr. Norton, has anyone looked at brain damage resulting from hypoxia caused by reduced oxygen concentration or from an exposure to a chemical that causes an equivalent methemoglobinemia to the carboxyhemoglobin level that you've studied?

DR. NORTON (University of Kansas Medical Center): I'm not aware of any studies specifically like ours. Not in the way that we have been doing it specifically. There have been quite a few studies on hypoxia, most often produced by enclosing a rat in a closed box and just letting it use all the air that's available. There has definitely been some damage reported in the fetus as a result of that treatment. There have been some studies on monkeys in which the hypoxia was produced by hypotension in the mother and by exteriorization of the uterine vessels at near term which were partially clamped to produce the hypoxia. Depending upon the type of hypoxic damage, ischemic or nonischemic, there are differences in the degree of injury. Carboxyhemoglobin hypoxia occurs in the presence of normal blood flow and the evidence is pretty good that if you maintain blood flow there is less damage from hypoxia than if you have associated ischemia where there is an accumulation of lactic acid and other substances. There has been considerable interest in hypoxia during birth in the human because of the potential for kinking of the umbilical cord and a relatively prolonged period of ischemia. A prolonged period of ischemia or a prolonged period of hypoxia is unquestionably damaging and we have evidence of that in the human as well as in monkeys and rats. There are no comparable studies looking at the sites of damage in the brain in the same way as we did. Most other work has been looking for brain necrosis which you can produce quite readily. There haven't been many studies that have looked at subtle damage to the brain after the brain has had an opportunity to recover from the anoxic episode.

DR. MERIGAN (University of Rochester): I'd like to ask Dr. Norton a question. Aultman has attempted to differentiate prenatal and postnatal neurogenesis on the basis of the type of neuron which is being formed and he has classified these as macro-neurons which occur prenatally and generally have long processes associated with them versus the micro-neuron which is the sort of inner neuron. Are there data that you're aware of which would argue that those two kinds of neurons are differentially sensitive to a given type of insult?

DR. NORTON (University of Kansas Medical Center): There's very little evidence that there's any unique sensitivity of the macro or micro neuron because the time of gestation at which the insult occurs is the determining factor in what kind of damage occurs. The brain is formed in a rostral direction, roughly speaking, so that damage during the formation of the brain early in gestation, if the rear end of the brain is damaged it results in a nonviable organism after birth and a relatively severe microcephalia. If, on the other hand the damage is more in the rostral direction, you get progressively more and more probability of the organism surviving. In our experience this does not appear to be related to a unique decimation of one specific population of neurons. Postnatally there is a clear opportunity to damage certain types of neurons. The small granule cells are formed in postnatal neurogenesis in both man, rat, and monkey. In those animals that are born in an atricial stage it's quite possible to interfere with cell division

postnatally and wipe out populations of granule cells in the hippocampus, the cerebellum or in the cerebral cortex. The finding of a selective agent that would wipe out small cells that are dividing and migrating from the ependyma has not been published and has not been proposed for any toxic agent that I'm aware of.

MR. JONMAIRE (Uniroyal Chemical Company): I'd like to ask Dr. Reiter a question about what the regulatory agencies plan to do with these screening systems that he has proposed. Could you tell us what they want to do with these systems and what they can do? Could you also tell me what to compare positive results with? When I say that, I mean, if something turns out to be behaviorally toxic, what do you compare that to, to make a regulatory decision?

DR. REITER (U. S. Environmental Protection Agency): I don't know a precise answer to your first question but I think that much of the current impetus for developing screening methodologies arises from the Toxic Substances Control Act. I think it's section four which regulates chemicals that are currently in commerce and section five deals with the introduction of new compounds into commerce. The Office of Toxic Substances has estimated that approximately a thousand new compounds are introduced into commerce each year. I think that the primary emphasis at this point is to develop methodologies which can be used to evaluate target organ toxicity at the screening level. Because the burden of proof is on industry, they have to provide the toxicity data on a new compound if they're going to introduce this compound into commerce. And I'm not as familiar with a lot of the legal aspects of the Act as I perhaps should be, but I think that's basically correct. Particular screening test procedures would be required by EPA to determine whether further information on the toxicity of these compounds is needed. The point I tried to make in my talk this morning is that when you use tests for behavioral effects, you really have a problem because at some level of exposure, every compound is going to be behaviorally toxic. Unless you set up some sort of criteria, whereby you will accept that data as a flag indicating that further testing is needed, then you might as well not do the primary screen. The only way I can see that that can be done is to try to juxtapose the dose which is behaviorally active to a dose which is producing overt toxicity. That ratio is what I called a behavioral toxicity index. Perhaps the ED<sub>50</sub> of the chemical isn't even affected because you may run into certain kinds of behavioral tests which have a floor effect. In other words, they might be very sensitive in detecting a 10% change of normal activity but you're never going to produce an effect of 40% reduction. The ED<sub>50</sub> may not be the appropriate way to go, but it's the way we chose to look at our initial data. You've got to be able to compare the dose which is behaviorally active to that which is producing overt toxicity. If that ratio is high, then it would suggest that the nervous system is a target organ for the chemical tested and I suppose that that would be grounds for requesting that more information concerning the specific neurotoxicity of that compound be provided before that compound is permitted into commerce. So I think section five of the TSCA is probably a lot easier to deal with than section four because section four deals with compounds that are currently in commerce and it's much more difficult to change the current standards

than to set them for new chemicals. The question you're asking is at what level of toxic effect are we going to regulate and that entails asking some very specific questions about the kinds of effects. In a more qualitative sense, what are the types of functional deficits that are produced by exposure and at what exposure level. This is the level of inquiry that requires the most sophisticated and sensitive techniques.



## SESSION II

### PULMONARY PHYSIOLOGY, BIOCHEMISTRY AND STRUCTURE DURING INDUCTION OF EMPHYSEMA-LIKE DISEASE IN EXPERIMENTAL ANIMALS

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EMPHYSEMA-LIKE DISEASE IN EXPERIMENTAL ANIMALS  
EXPOSED TO OXIDANT GASES

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Early in the last century, Laennec described pulmonary emphysema and included as characteristics, interference with airflow from alveoli to airways and a loss of alveolar elasticity (Laennec, 1835).

Some of the more critical observations about emphysema since then have been epidemiologic -- first anecdotal and then rigorous (Hammond, 1966; DHEW Publication, 1979). They established its strong correlation with the inhalation of tobacco smoke and led investigators to a tangible understanding of chronic obstructive disease. For a long interval, the intimate acquaintanceship with emphysema was shared between its victims and pathologists. Contributory etiologic factors and pathogenesis were unveiled stepwise, many as measurable deficiencies in function. Early on, these were often inconsistent and confusing; now clinical and academic physiologists describe respiratory function in its various dimensions with considerable confidence.

In recent years the oxides of nitrogen ( $\text{NO}_x$ ) were identified as components-in-common in several industrial and occupational atmospheres, in fumes of fermenting silage, then in photochemical smog (World Health Organization, 1977) and also in tobacco smoke (Haagen-Smit et al., 1959).  $\text{NO}_x$  often induces overt symptoms and also disease of the respiratory tract of various kinds and degrees of reversibility (World Health Organization, 1977). Less commonly, ozone ( $\text{O}_3$ )--one of the important atmospheric oxidants, and more than ten times as toxic as nitrogen dioxide ( $\text{NO}_2$ ) (Freeman, Unpublished Data)--is also known to provoke disease in certain occupations (World Health Organization, 1978). However,  $\text{O}_3$  is practically nonexistent in tobacco smoke (DHEW Publication, 1979). On the other hand,  $\text{NO}_2$ , the most toxic of the nitrogen oxides, is reputed to contribute up to 10% of the hundreds of ppm of  $\text{NO}_x$  in tobacco smoke (Haagen-Smit, 1969). This is at least ten-fold its peak ambient concentration, even in heavy smog (World Health Organization, 1977). Therefore, in 1961, we selected  $\text{NO}_2$  as a likely initiator of an emphysema-like disease in the rat, using both ambient concentrations and those in the range found in tobacco smoke (Freeman and Haydon, 1964). [Parenthetically, there is little evidence that nonsmokers residing in smoggy areas have more emphysema than do equivalent residents of nonpolluted areas (S. Ishikawa et al., 1968). This is true despite the combined presence of  $\text{NO}_2$  and  $\text{O}_3$  in photochemical smog, both gases often

achieving similar daily peaks a couple of hours apart (U. S. Environmental Protection Agency).] Incidentally, high ambient concentrations of  $O_3$  alone can induce emphysema in rats upon prolonged daily exposure (Stokinger et al., 1957), whereas ambient levels of  $NO_2$  do not. I shall dwell on  $NO_2$  because, first, it relates directly to our subject, emphysema, and, second, atmospheric  $O_3$  depends upon the prior presence of  $NO_2$  in the photochemical cycle.

It became clear by 1963 (Freeman and Haydon, 1964) that young growing rats exposed continuously in our laboratory to greater than 10 ppm  $NO_2$  would slowly and insidiously develop an emphysematous disease and, in about six months, would suffer severe respiratory distress, would gain weight at a reduced rate and, during their growth, would develop an enlarged thorax with a marked dorsal kyphosis of the thoracic spine (Freeman et al., 1968) (Figures 1 and 2). Upon exposing their lungs to the atmosphere, they fail to collapse as do normal lungs. They were air-filled but not edematous--wet-dry weights of these lungs being no different from normal (Freeman et al., 1968) (Table 1). Exposed lungs were larger and heavier and had less circulating pulmonary blood--determined as Fe per gram of lung--compared to normal lungs of the same age. They tended to resist compression and filled irregularly and relatively slowly with fluid through the trachea, as though both the flow of air from the alveoli and fluid into the alveoli were restricted. Accordingly, minimal or residual volume at atmospheric pressure was clearly greater than normal. Also, under a standard pressure of 25 cm  $H_2O$ , their volumes were considerably greater. Apparently, compliance was increased in such lungs. See Figure 3.



Figure 1. Normal and chronically exposed rats (to  $NO_2$ ) with their excised lungs at atmospheric pressure. Note enlarged volume of lungs and thorax in exposed rat.

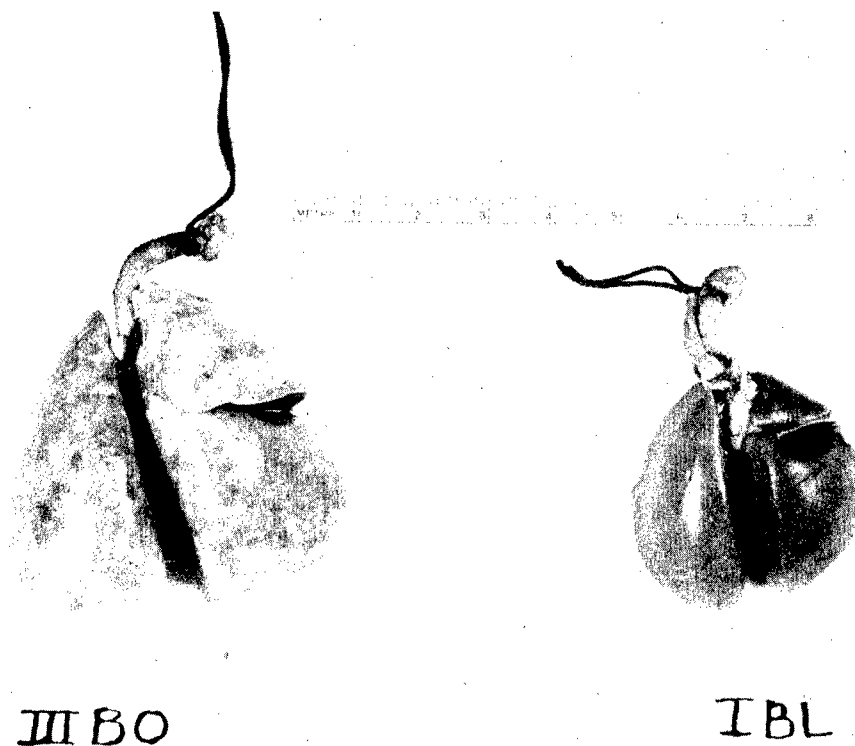


Figure 2. Lungs, as in Figure 1, distended to 25 cm H<sub>2</sub>O pressure.

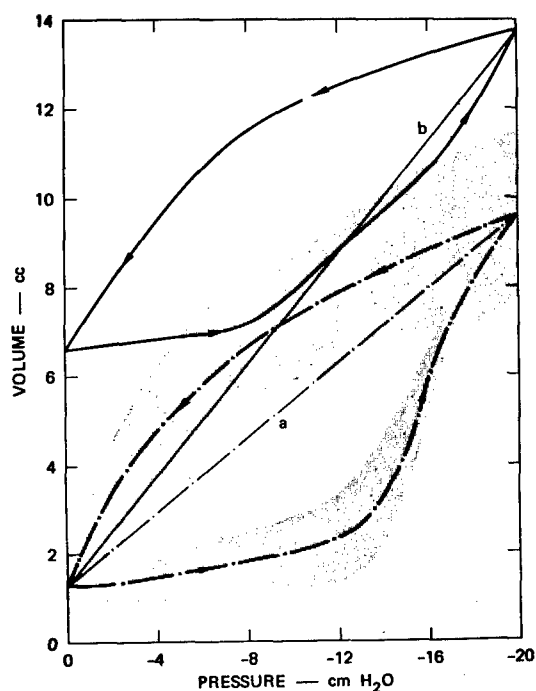


Figure 3. Pressure-volume curves. Slope "b" is comparative specific compliance of emphysema-like lung.

TABLE 1. COMPARISON OF WET AND DRY WEIGHTS OF  
NORMAL AND EMPHYSEMATOUS RAT LUNGS

Experimental Regimen	Wet Weight (g)		Dry Weight (g)		Dry $\times$ 100 Wet	% Loss on Drying
	$\bar{x}$	$\pm$ SD	$\bar{x}$	$\pm$ SD		
Control (n = 2)	1.86	0.95	0.46	0.04	24.7%	75.3
Emphysematous (n = 5)	4.00 (+115%)	0.48	0.95 (+106%)	0.10	23.7%	76.3

Early measurements in vivo of transpulmonary resistance and compliance, using a modified Amdur and Mead method (Amdur and Mead, 1958) in the rat, were not revealing, despite the abnormal behavior of the excised lungs and the more compelling microscopic evidence of narrow and inflamed terminal bronchioles and of distended alveoli and their attenuated septal walls.

On closer inspection, the "small airways" and the acini--composed of terminal bronchioles with their short respiratory portions--alveolar ducts, and adjacent alveoli all contain aggregations of macrophages, mainly, and some mononuclear and polynuclear inflammatory cells (See Figure 4a,b). These cells are found among the cellular debris, fibrinous exudate, and mucinous secretions. The epithelial cells lining the acini are larger than normal and their bronchiolar and ductal smooth muscle and connective tissue are hypertrophic (Freeman and Haydon, 1964) (See Figure 5). Each of these factors adds to potential obstruction. More peripherally, on the contrary, the parenchyma is distended, alveolar septa are attenuated, and some appear to be disrupted (See Figures 6 and 7). To quantify the apparent loss of parenchymal structure, rats were allowed to survive for at least two years by alternating periods of recovery, after the initial six months, with additional periods of exposure, so that the pathogenesis could be protracted for the usual life expectancy of the rat. The total number of alveoli in the enlarged lungs of such rats was reduced about in half, depriving them of an appreciable amount of gas-exchanging surface area (Freeman et al., 1972) (Table 2). From this, we can infer a loss of alveolar septa--the *sine qua non* of emphysema, as defined by committee action of the American Thoracic Society (1962).

In 1965 Ericksson, from Laurell's laboratory, reported a congenital deficiency in  $\alpha_1$ -antitrypsin that was associated with the propensity for developing emphysema, as exemplified by their parallel prevalence in affected families (Ericksson, 1965). The observation stimulated intensive ongoing research on the balance between proteolytic enzyme activities and their natural inhibitors, which might be upset by enzyme-discharging inflammatory cells. Macrophages and granulocytes would have to be attracted secondary to initial injury from exogenous sources (Pulmonary Emphysema and Proteolysis, 1972). Such imbalance is suspected of inviting digestion of connective tissue, especially the elastin of the parenchyma, even in the 85 to 90% of the emphysematous population that is capable of producing "normal" amounts of  $\alpha_1$ -antitrypsin (Lunan & Freeman, 1972).

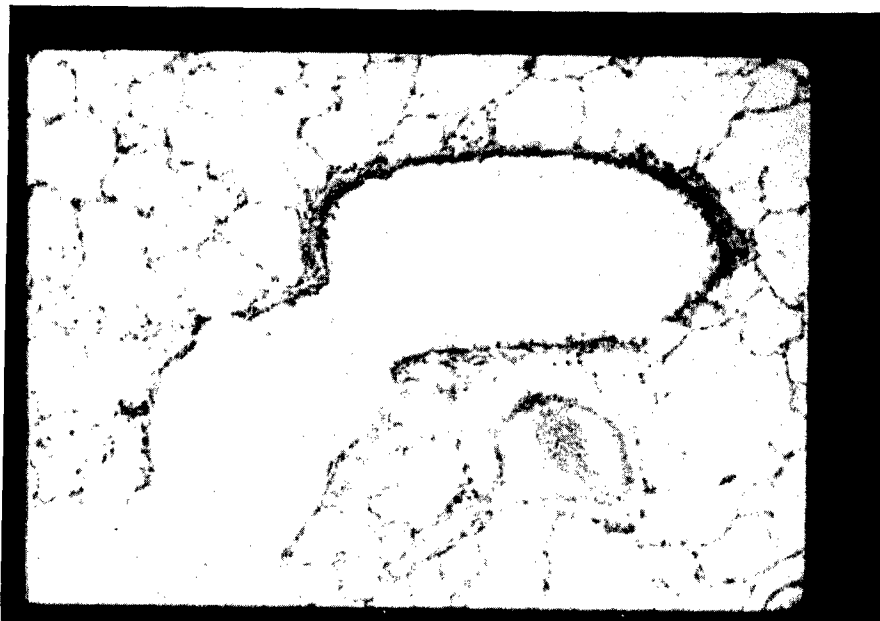


Figure 4a. Terminal bronchioles of control lungs with NO<sub>2</sub>-induced chronic bronchiolitis. (Magnified 200x)

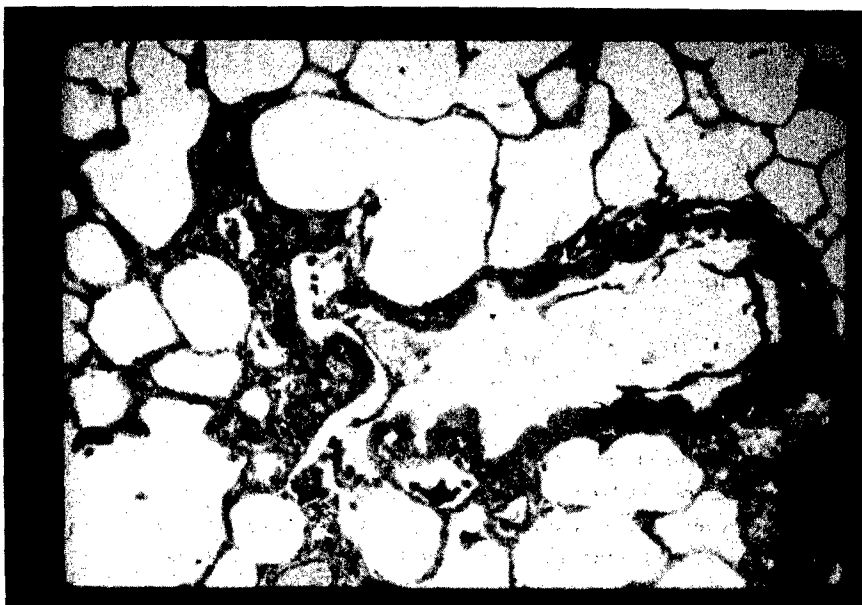


Figure 4b. Terminal bronchioles of exposed lungs with NO<sub>2</sub>-induced chronic bronchiolitis. (Magnified 200x)

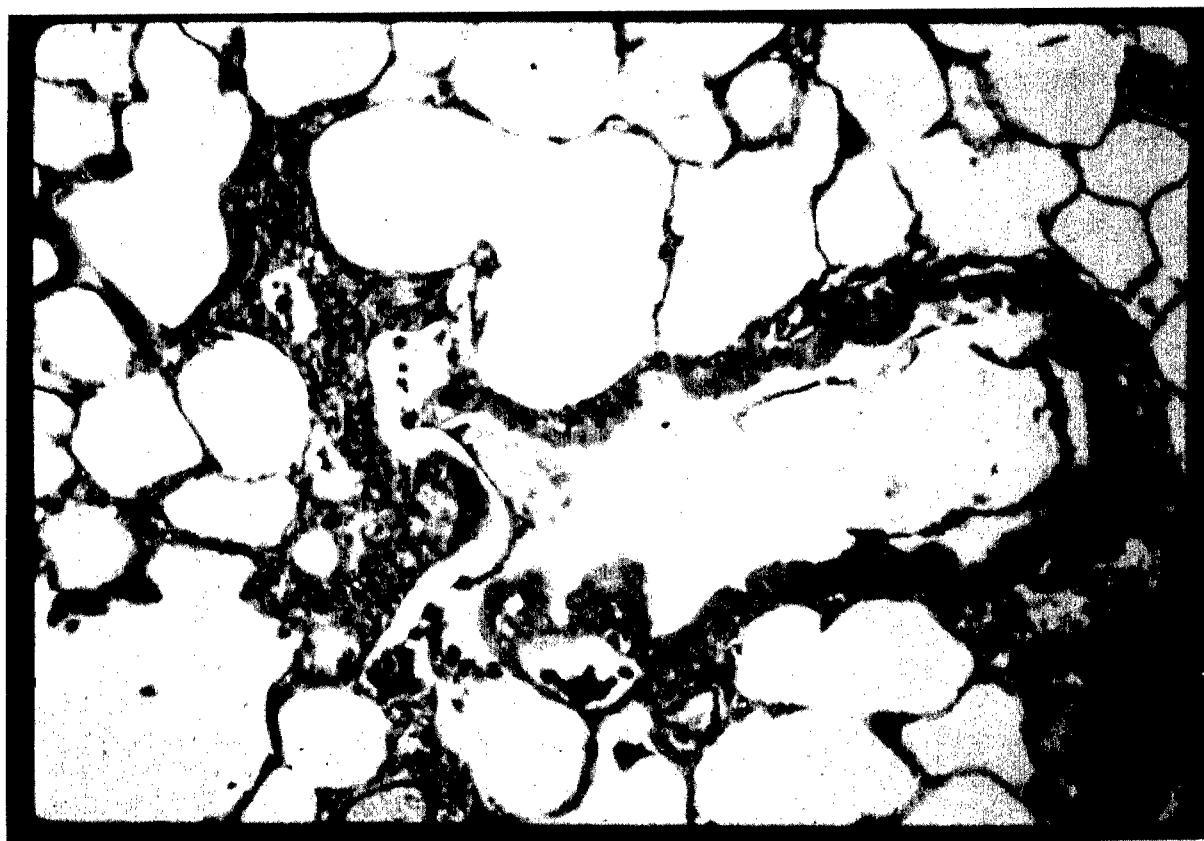


Figure 5. Higher magnification (320x) of exposed bronchiole of Figure 4b.

TABLE 2. RELATIVE NUMBERS OF ALVEOLAR SPACES IN A  
TYPICAL NORMAL AND TYPICAL NO<sub>2</sub>-EXPOSED  
LUNG IN CROSS SECTION\*

	Control	Experimental
Squares counted	26	26
Alveoli per squares (mean $\pm$ SD)	78 $\pm$ 17	36.7 $\pm$ 10 (39.6 $\pm$ 10) <sup>†</sup>

\*Maximal cross sectional areas cut 4- $\mu$  thick.

<sup>†</sup>Corrected for difference in cross sectional areas between mean value of control lungs and of the typical NO<sub>2</sub>-exposed lung used. The difference was determined by counting the total number of squares covering each lung. Instead of equal numbers of squares, 8 per cent more were required to cover the NO<sub>2</sub>-exposed lung.

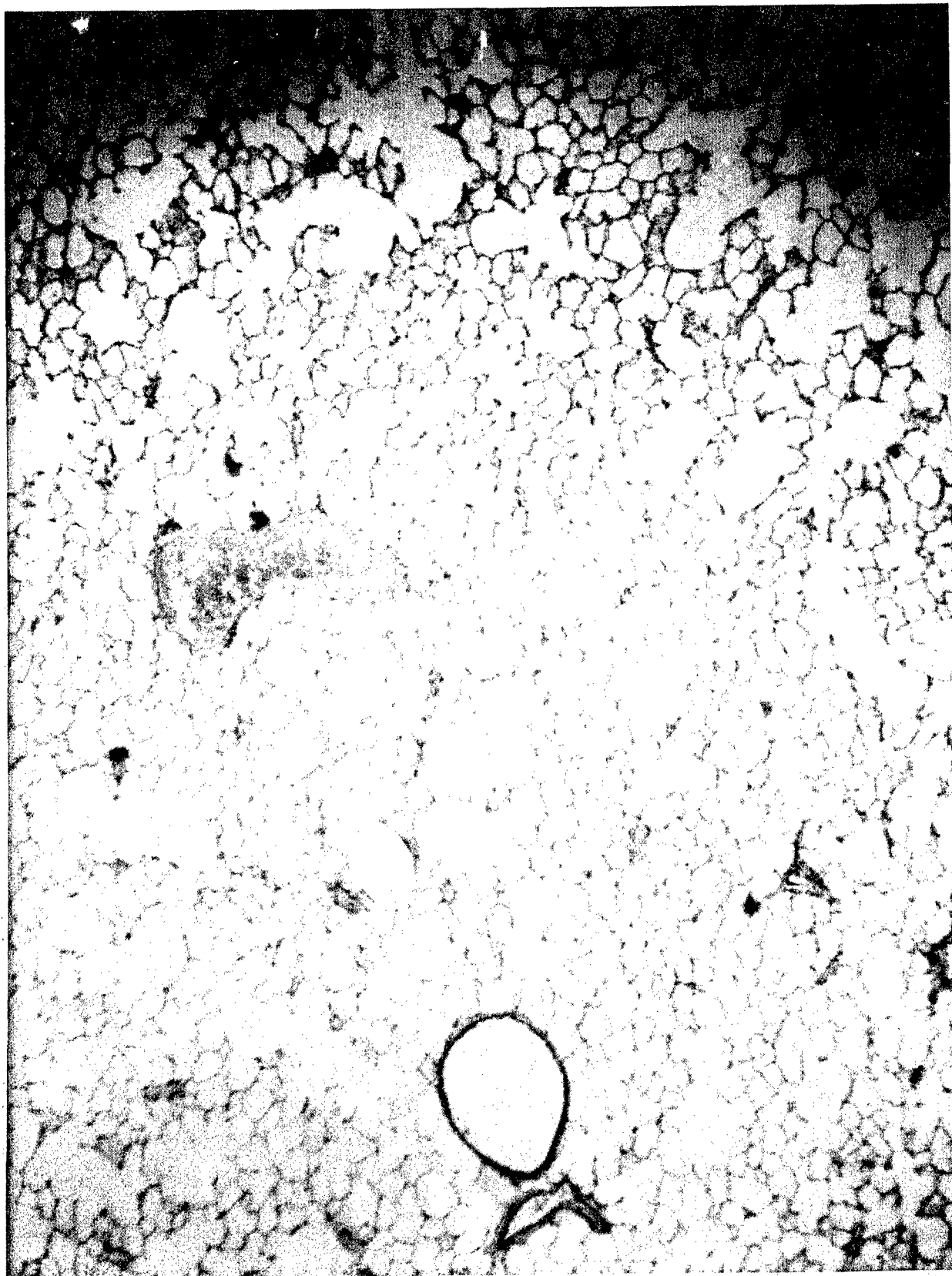


Figure 6. Nonexposed control parenchyma for Figure 7. (Magnified 25x)





Figure 7. Parenchyma of rat's lung following prolonged exposure to about 20 ppm  $\text{NO}_2$ . (Magnified 25x)

In our initial studies, we observed, by their staining properties (Freeman and Haydon, 1964) and electron microscopy (American Thoracic Society, 1962) along with others, regular morphologic changes in the thickness and continuity of both collagen and elastin and have since then measured the diameters of such fibers in the alveolar ducts where they encircle the airways together with smooth-muscle (See Figures 8 through 10). Both types of fiber proved to be broader in  $\text{NO}_2$ -exposed rats at sites in common with age-controlled normal rats (Figure 11). Total collagen, extracted from exposed lungs, increased about 20% by the end of a year while Type III was reduced about 14% (Freeman, Unpublished Data). [An amino acid profile of extracted elastin, however, was essentially identical to the normal extract; the total amount was not determined (Freeman, Unpublished Data).]

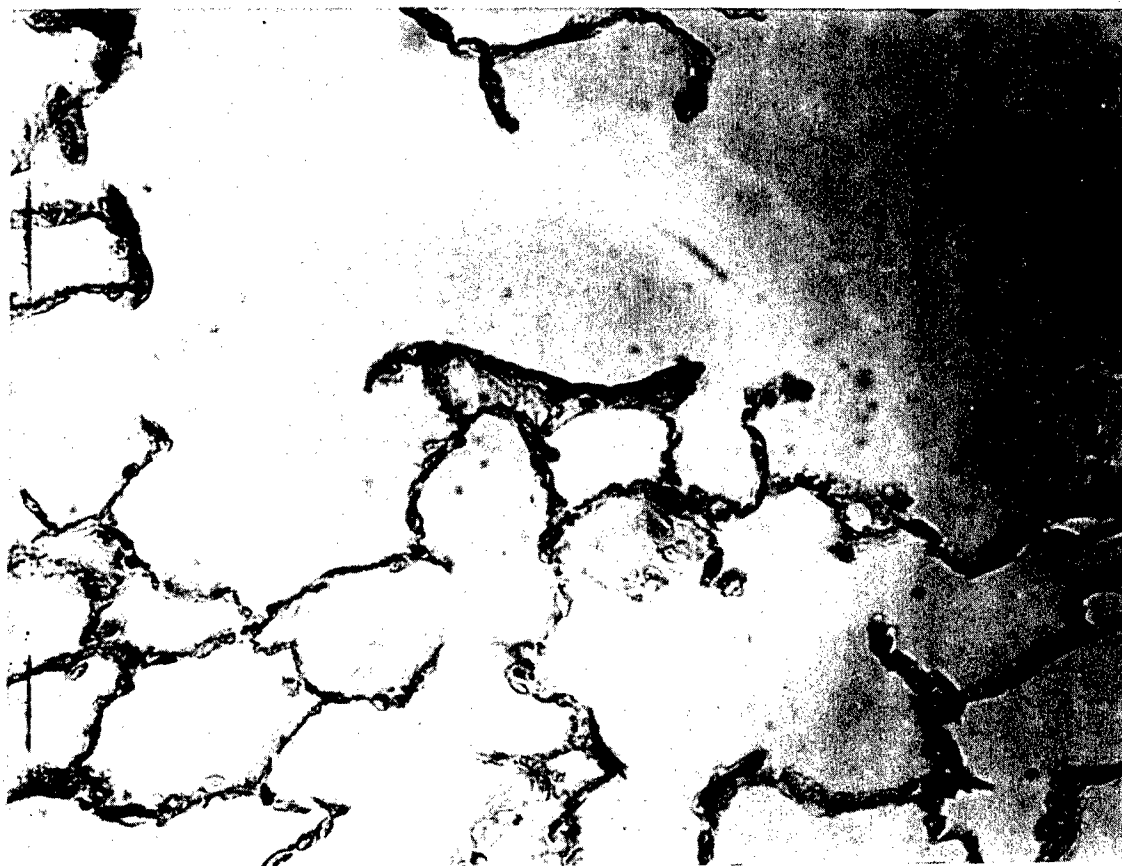


Figure 8a. Collagen, stained with ABOG, in alveolar ducts of normal rat lungs.  
(Magnified 320x)

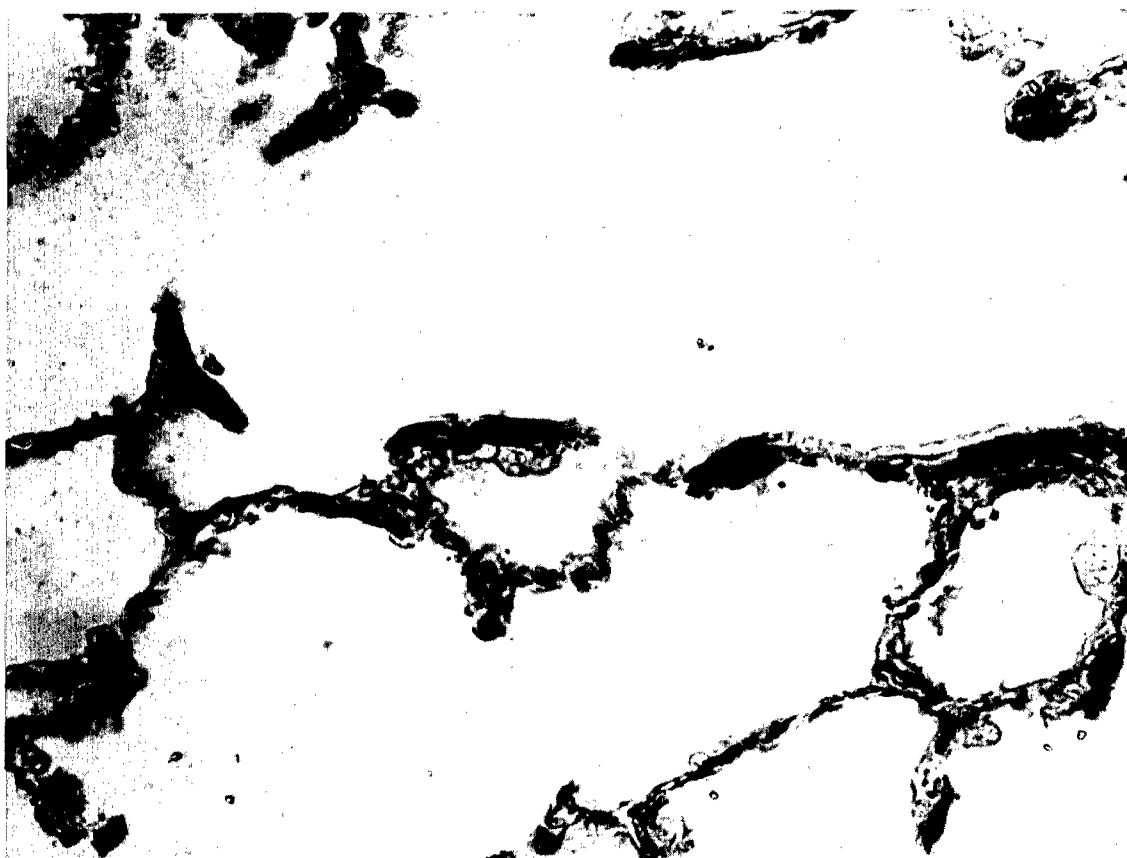


Figure 8b. Collagen, stained with ABOG, in alveolar ducts of chronically exposed rat lungs. (Magnified 320x)

In 1970, we measured proteolytic activity in lungs in situ at various times during exposure to about 17 ppm  $\text{NO}_2$  by pouring the chromogenic substrate, Tris- $\alpha$ -N-benzoyl-DL-arginine-p-nitroanalide (BAPA) into the lungs, and discovered a significant elevation in proteolytic activity and its return to baseline during that period (Lunan and Freeman, 1972) (See Figure 12). The peak values coincide with the initial injury and desquamation of Type 1 alveolar cells to expose the basal membrane and probably facilitate leakage of plasma. (See Figure 13.) Rapid repair follows as determined by the division of tritium-labelled Type 2 cells (Evans et al., 1971). (See Figure 14.) The rapidity by which proteolytic activity followed exposure suggests that enzymatic activity may have been derived largely from ruptured epithelial cells and leakage of blood, rather than from inflammatory cells. Another obviously effective agent that can, indeed, destroy parenchymal cells and tissues is the  $\text{NO}_2$  itself, or its reaction product(s) (Freeman, Unpublished Data).



Figure 9. Same as Figure 10. Unexposed lung. (Magnified 504x)



Figure 10. Acid orcein stain of elastic fibers in NO<sub>2</sub>-exposed lung. (Magnified 504x)

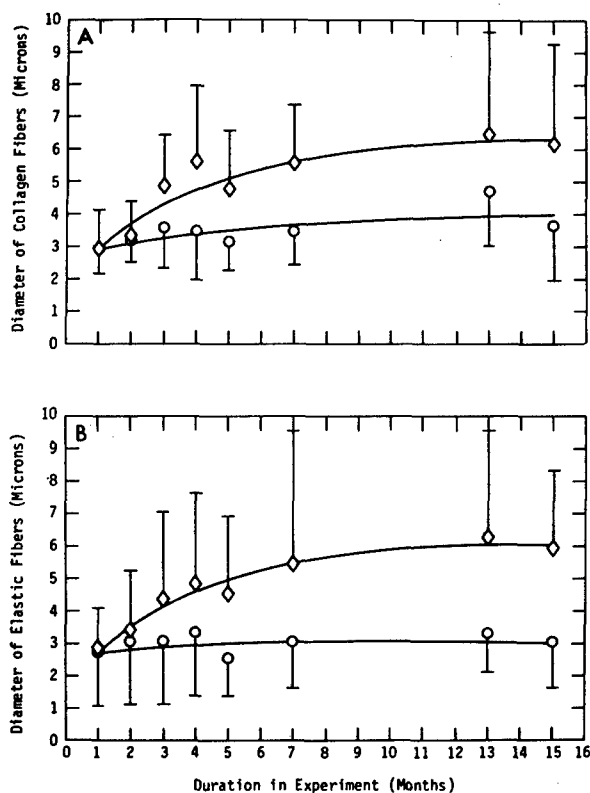


Figure 11. Comparative widths of collagen and elastin fibers as a function of duration of exposure to  $\text{NO}_2$  in alveolar ducts. Control and exposed lungs.

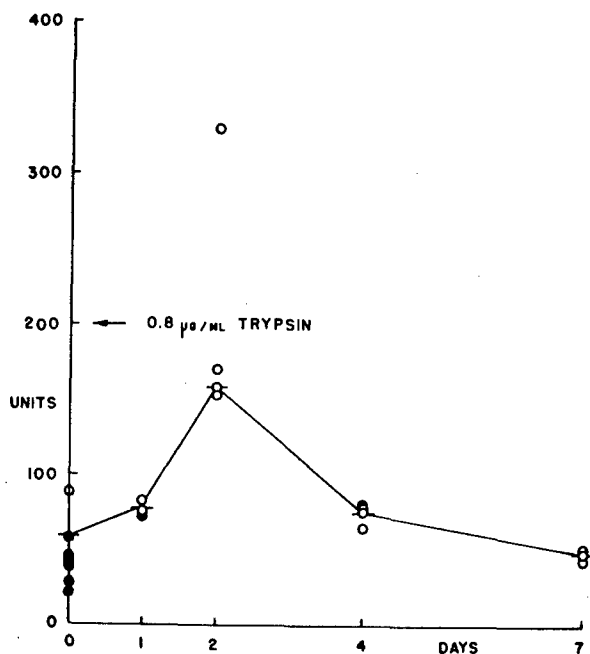


Figure 12. Proteolytic activity in lung as a function of duration of exposure to  $\text{NO}_2$ .



Figure 13. Desquamating Type 1 alveolar cells following exposure to  $\text{NO}_2$ .  
(Magnified 504x)

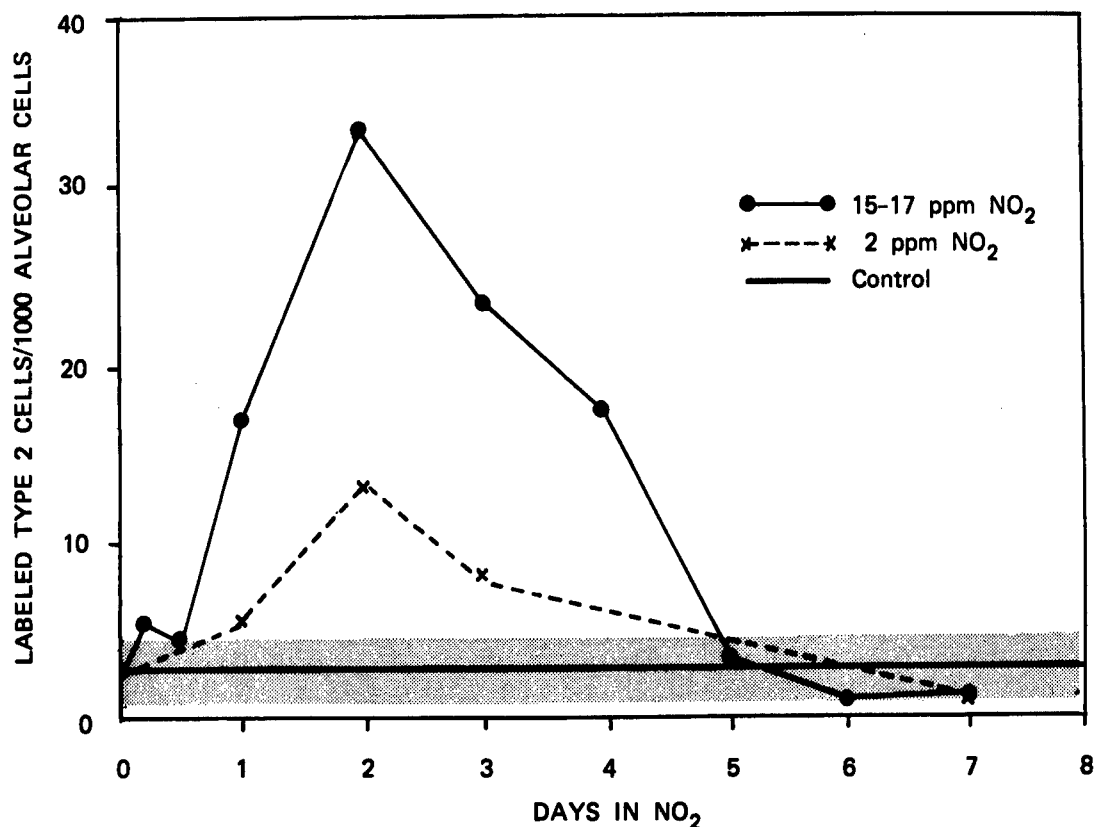


Figure 14. Replication rates of labelled Type 2 cells as a function of duration of exposure to NO<sub>2</sub>.

When homogenized rat lungs were exposed to a high concentration of NO<sub>2</sub> labelled with the nonradioactive isotope N<sup>15</sup>, (<sup>15</sup>NO<sub>2</sub>) was found to be incorporated largely in the extractable lecithins and partially in protein (Freeman, Unpublished Data). This was observed in a collaborative study with Carter at Oak Ridge National Laboratories.

A third factor may simply be expiratory air pressure (Juhos et al., 1980), or such stress combined with direct chemical or enzymatic weakening of alveolar structure, or all three. While the lungs of continuously exposed rats are increasing in volume and weight, their terminal bronchiolar airways, as measured at a precise point (Figure 15) diminish in diameter about 44% during a year or more of residence in 15 ppm NO<sub>2</sub>. This is equivalent to a 69% reduction in its cross-sectional lumenal area (see Figure 16 and Table 3). Thus, small airway disease or bronchiolitis most likely plays a major role in this form of experimental emphysema (Hogg et al., 1968) as it appears to in the human disease (Thurlbeck, 1976) and is often manifest through frequency dependence of compliance (Figure 17) or by closing volume determination.



**A**

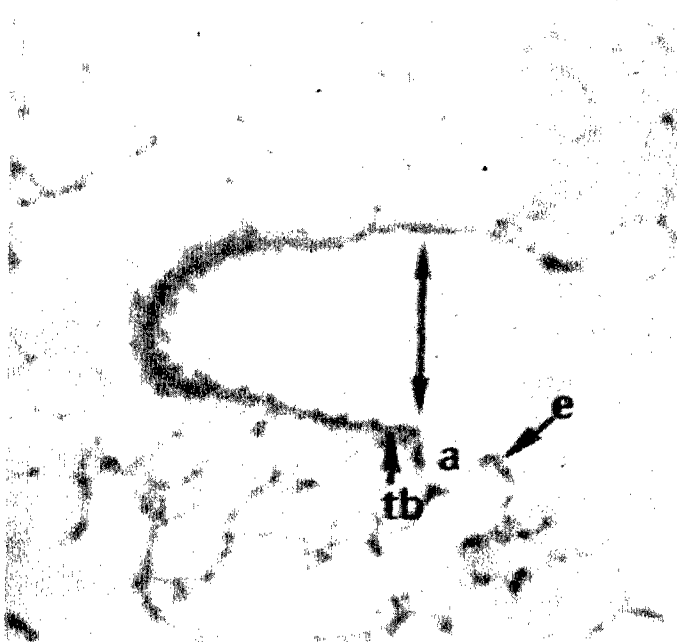


Figure 15a. Sites of measurements of diameters in small airways. (Magnified 200x)

**B**



Figure 15b. Sites of measurements of diameters in small airways. (Magnified 200x)

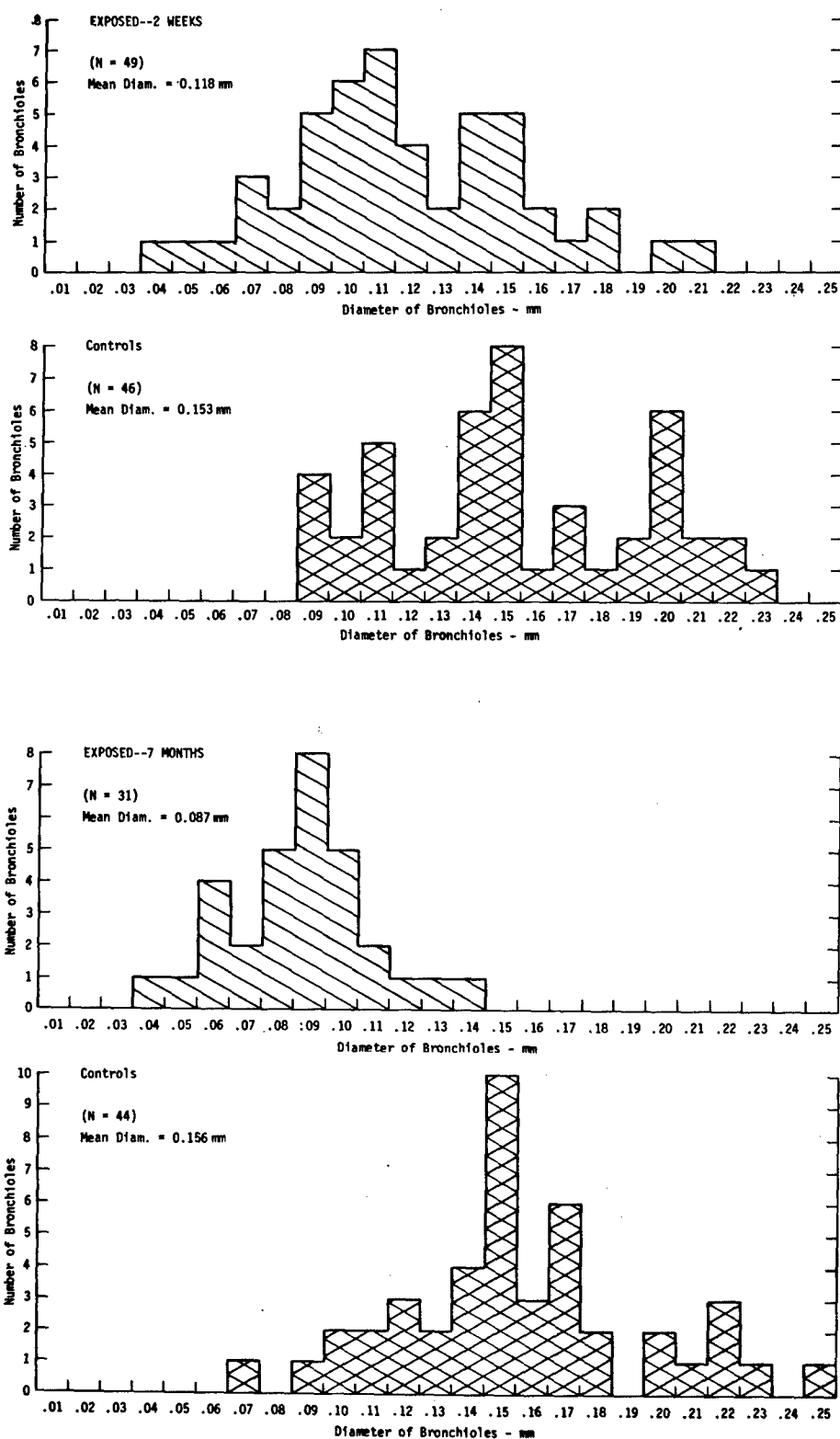


Figure 16. Comparative distributions of terminal bronchiolar airway diameters of normal and chronically exposed lungs.

TABLE 3. REDUCTION IN COMPUTED FLOW RATES RESULTING FROM THE CONSTRUCTION OF LUMENAL AREA IN THE TERMINAL BRONCHIOLES IN NO<sub>2</sub>-EXPOSED RAT LUNGS

Exposure Period	Control		Exposed			
	Mean	Flow Rate	Mean	Flow Rate	Reduction	Flow
	Area of	Through	Area of	Through	in	Remaining
	Lumen ( $\mu^2$ )	Lumen (cc/sec)	Lumen ( $\mu^2$ )	Lumen (cc/sec)	Area (%)	(% of control)
1 Week	15,386	6.1	10,930	3.1	29.0	50.3
2 Weeks	18,376	8.7	10,930	3.1	40.5	35.3
3 Weeks	17,195	7.6	11,304	3.3	34.3	43.2
1 Month	19,104	9.4	10,382	2.8	45.7	29.6
2 Months	22,420	12.9	9,847	2.5	56.1	19.3
3 Months	23,223	13.8	7,386	1.4	68.2	10.1
4 Months	21,113	11.4	8,167	1.7	61.3	14.9
5 Months	20,857	11.2	7,085	1.3	66.0	11.6
7 Months	19,104	9.4	5,942	0.9	68.9	9.7
13 Months	18,617	8.9	5,942	0.9	68.1	10.2
15 Months	21,371	11.7	6,644	1.1	68.9	9.6
17 Months	20,096	10.4	5,942	0.9	70.4	8.8

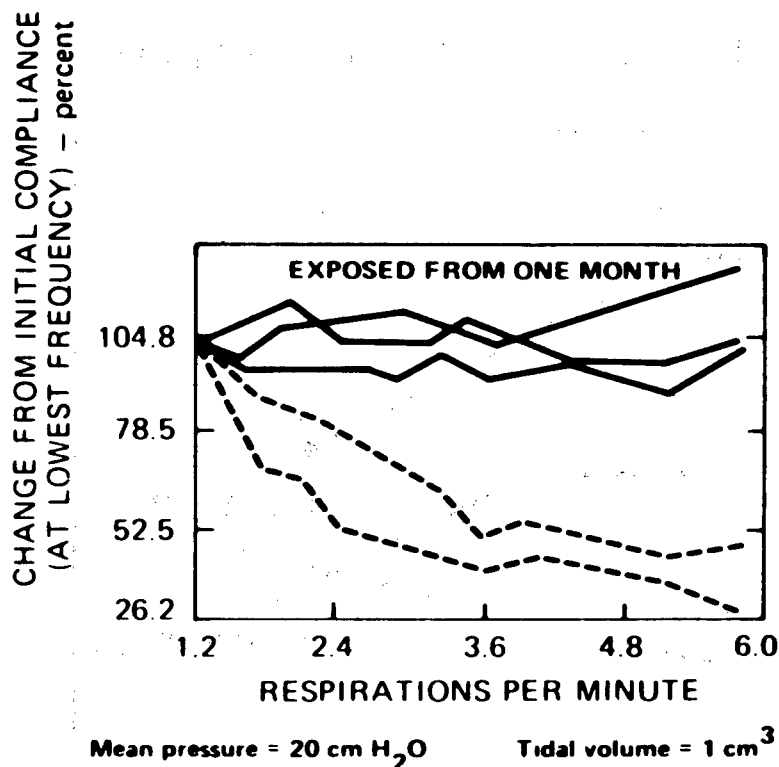


Figure 17. Comparative compliance curves as a function of frequency of respiration in normal and chronically exposed rats.

This oxidant-induced emphysema in the rat is accompanied by extra-pulmonary expressions, also characteristic of the human disease. For example, the heart weight increases, although we have not examined this in terms of cor pulmonale (Freeman, Unpublished Data). The arterial  $P_{O_2}$  becomes increasingly reduced and erythropoiesis is stimulated. The polycythemia then tends to adapt over several years of exposure in the monkey (Freeman, Unpublished Data). See Figure 18. Also, isoelectric focusing on polyacrylamide gel reveals a strong band of methemoglobin in the blood of exposed rats when compared to normal blood (Freeman, Unpublished Data). Species idiosyncrasies are revealed in the rat and in the primate (*Macacus speciosa*) also, as a tendency for red cells to be released with a somewhat reduced mean corpuscular volume during their accelerated generation. Therefore, the erythrocyte concentrations rise while the animals maintain stable hematocrits and normal mean corpuscular hemoglobin concentrations (Furiosi et al., 1973). As a result, the total surface area for gas-exchange in the red cell mass increases by the distribution of cellular hemoglobin into smaller packages.

Observations we have made in monkeys (*M. speciosa*) exposed to  $NO_2$  for up to 10 years (Freeman, Unpublished Data) tend to confirm our findings in rats and help to bridge the gaps in our knowledge about differences among species, particularly between experimental emphysema in the rat and its natural history in man.

## ERYTHROCYTE LEVELS IN NO<sub>2</sub>—EXPOSED MONKEYS

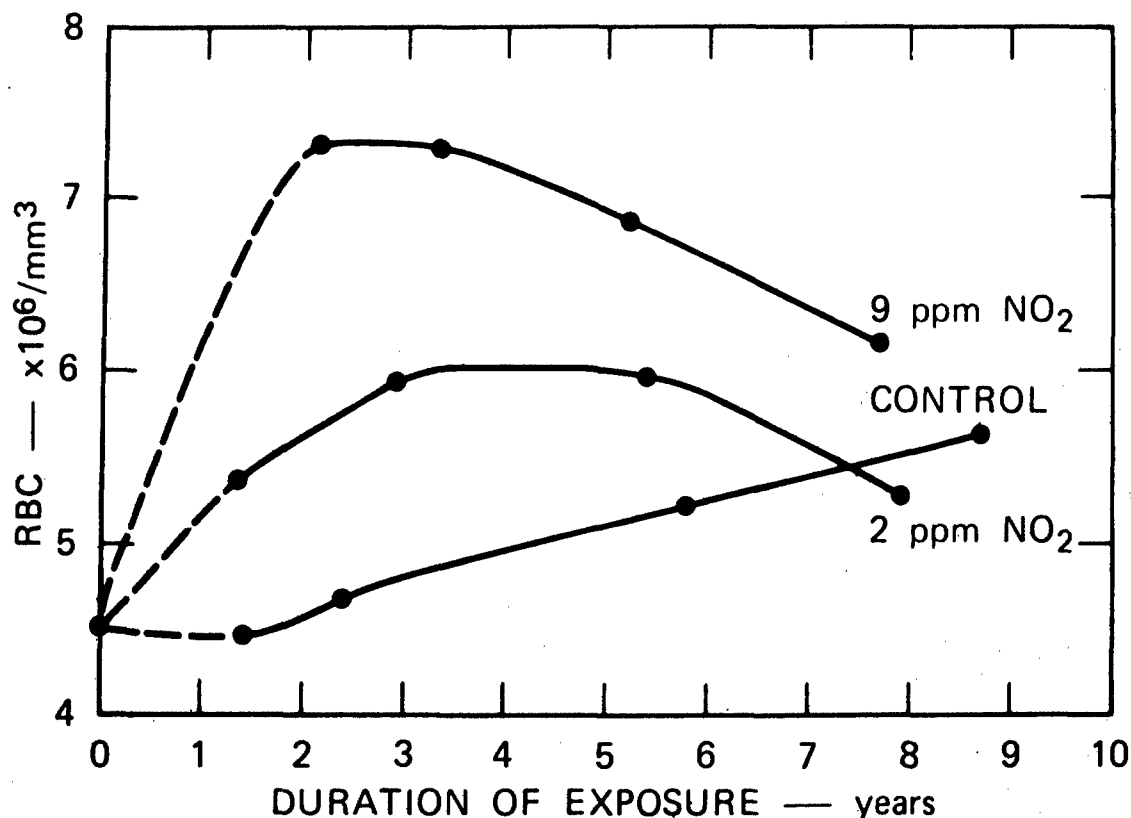


Figure 18. Erythrocyte concentrations as a function of duration of exposure of monkeys to NO<sub>2</sub>, and their controls.

Thus, the high concentrations of NO<sub>2</sub> in tobacco smoke, its conspicuous retention (> 80%) when inhaled, the unequivocal correlation between smoking and emphysema, and the several equivalent features in the pathogenesis in man, monkey, and rat implicate NO<sub>2</sub> as a major etiologic factor in emphysema in man as well as in the rat. Other components of tobacco smoke may also contribute to features unique to the human disease, such as phagocytized carbon particles. Also, the cadmium in tobacco smoke has been shown to injure lungs (Hayes et al., 1976).

In conclusion, the NO<sub>2</sub>-produced emphysema in the rat may be a first-cousin to smokers' emphysema in man, and provides a useful model in which to study etiology and details of its pathogenesis.

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#### ACKNOWLEDGEMENTS

Major contributors to this research were A. E. Brandt, S. C. Crane, M. J. Evans, N. J. Furiosi, G. B. Haydon, L. T. Juhos, K. D. Lunan and R. J. Stephens. I am grateful for the participation of many others.

This research was supported by grants from the Institute of Environmental Health Sciences and the Heart, Lung and Blood Institute of the National Institutes of Health, and by contracts from the Health Hazards Branch of the U. S. Environmental Protection Agency.

PULMONARY FUNCTION CHANGES IN SMALL ANIMALS FOLLOWING  
EXPOSURE TO OXIDANT GASES AND IN THE PRESENCE  
OF EXPERIMENTAL EMPHYSEMA

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INTRODUCTION

What are the consequences of chemically produced pulmonary damage in experimental animals or of the inhalation exposure of animals to environmental pollutants? Evaluation of the health effects of inhaled pulmonary toxicants is based on an understanding of normal lung function and an ability to determine both the presence of disease and the extent of such disease. Because of the diversity in disease processes, interpretation of pulmonary function tests is complicated. Different lesions affect the lungs in different ways. Therefore, tests of spirometry, ventilation, gas exchange, and pulmonary mechanics should be done.

Application of these tests to problems of environmental or occupational health will be useful to determine the nature and extent of pulmonary damage following inhalation exposure of the animals to pulmonary toxicants. Disease progression can be followed. Interpretation can be based on disease states as identified morphologically. In addition, these tests can be used to scale pulmonary function in animals of different body mass. Such information may be of importance in extrapolating effects of inhaled compounds on humans.

TESTS OF PULMONARY FUNCTION USED IN SMALL ANIMALS

Small animals present many advantages for environmental or toxicologic studies when compared to larger animals (less expensive, easier to expose, logistically less complicated). However, as one attempts to study smaller animals, the difficulty of accomplishing a given pulmonary function test

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increases. Respiratory events occur more rapidly and signals are smaller in small animals. Rapidly occurring events, including normal breathing, have higher frequency content in smaller animals. The frequency response of the measuring equipment should be tested and should be linear throughout the range of frequencies likely to be encountered. The requirement for stable, sensitive, and rapidly responding equipment may become a limiting factor.

Equipment dead space requires innovative techniques to avoid masking signals or changing respiration. Use of smaller tubing will decrease dead space, but, at the same time, will increase inertance in the tubing. An appropriate compromise needs to be found. In spite of these difficulties, a number of useful tests have been developed which can be used with small animals and are described here. All of these tests (except for the measurement of closing volume) have been successfully accomplished in animals as small as the 25 g mouse.

#### TOTAL LUNG CAPACITY AND RESIDUAL VOLUME

Lung volumes are likely to change in predictable ways as pulmonary disease develops. For example, both total lung capacity (TLC) and residual volume (RV) will increase in the presence of emphysema and both will decrease in the presence of restrictive disease. These subdivisions of lung volume are easy to measure and do not require extensive equipment. The TLC is determined by independently measuring the RV and the vital capacity (VC). The VC can be defined as the volume necessary to inflate the lungs from an airway pressure ( $P_{aw}$ ) of -15 to +30 cm H<sub>2</sub>O relative to atmospheric pressure ( $P_B$ ); that is, the volume to inflate the lungs from RV to TLC. This volume change can be measured in a plethysmograph or as the displacement from a syringe as the lungs are inflated.

The RV can be measured by either of two methods. Gas dilution techniques suitable for use with small animals have been described by Takezawa and his co-workers (Takezawa et al., 1980). They are easy to use and do not require extensive measuring equipment. The gas in the lung is equilibrated with a test gas of known volume which contains 0.5% neon (Ne). The change in Ne concentration is proportional to the lung volume into which the test gas has been diluted. Another technique used to measure RV involves the use of a sensitive plethysmograph. By briefly occluding the airway at RV and then measuring the changes in  $P_{aw}$  and the change in thoracic gas volume ( $V_{TG}$ ), it is possible to calculate the RV using Boyles Law (Koo et al., 1976; Sinnett et al., 1981). A correction can be applied to this measurement to account for the oxygen absorbed during the procedure. These two methods for measuring RV are equivalent in small animals.<sup>1</sup>

#### SINGLE BREATH DIFFUSING CAPACITY FOR CARBON MONOXIDE

The  $DL_{CO}$  is likely to be reduced when ventilation and blood flow are not matched or when alveolar walls are damaged such as occurs in the presence of

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<sup>1</sup>James Raub and Robert Mercer, unpublished observations.

emphysema. This test is useful to predict changes from normal gas exchange. It is easy to perform and, like the measurement of lung volumes by gas dilution, does not require extensive equipment. Methods for the measurement of the diffusing capacity for carbon monoxide ( $D_{LCO}$ ) have been reported by Takezawa et al. (1980). These methods involve inflating the lungs with a test gas containing 0.5% Ne and 0.5% carbon monoxide (CO) for a 10 second period of breath holding. At the end of the breath hold, about 50% of the gas is removed from the lungs to wash out the dead space and the remainder of the gas is immediately removed as a representative alveolar sample. Ne and CO concentration are determined with a gas chromatograph, and the rate of CO diffusion from the alveoli into the blood can be calculated.

#### MULTIPLE BREATH NITROGEN WASHOUT

This test involves rather extensive equipment including a plethysmograph, recorders, and a  $N_2$  meter. Data analysis can be greatly facilitated by using automatic data processing equipment which allows the use of more rigorous methods such as moment analysis (Saidel et al., 1975). The multiple breath nitrogen washout promises to be useful especially in cases where "slow compartments", that is poorly ventilated units, exist.

This test is performed by ventilating an animal with 100%  $O_2$  and measuring the airway  $N_2$  concentration (Holub and Frank, 1979). With each breath, the  $N_2$  remaining in the lungs is diluted until it is completely washed out of the air spaces and only that  $N_2$  diffusing from tissue stores appears in the expired gas. A typical tracing of a multiple breath nitrogen washout is the control tracing of Figure 1. If there are slow compartments in the lung, it will take longer to washout the  $N_2$ . As a first approximation, we have calculated  $\log \% N_2$  versus breath number and measured a slope for  $N_2$  washout ( $\log \% N_2/\text{breath}$ ). In addition, the number of breaths required to reach a  $N_2$  concentration of less than 1% has been called the breath index.

#### VOLUME - PRESSURE RELATIONSHIPS

The quasi-static compliance characteristics of the respiratory system require specialized equipment. However, it is a useful test which has been used by Snider and his co-workers to evaluate the presence of pulmonary disease (emphysema) in small animals (Snider et al., 1977). The  $P_{aw}$  and the changes in  $V_{TG}$  must be measured. The latter is conveniently accomplished in a plethysmograph. Compliance can then be determined from the least-squares regression fit of the deflation limb of the pressure-volume curve. Since the chest wall is exceedingly compliant in small animals, a change in total respiratory system compliance will approximate changes in lung compliance. However, lung compliance can be separated from chest wall compliance by measuring pleural pressure. A fluid-filled esophageal catheter, properly placed in the esophagus, will follow changes in pleural pressure closely. Koo and his co-workers have described some elegant techniques to accomplish these measurements (Koo et al., 1976).

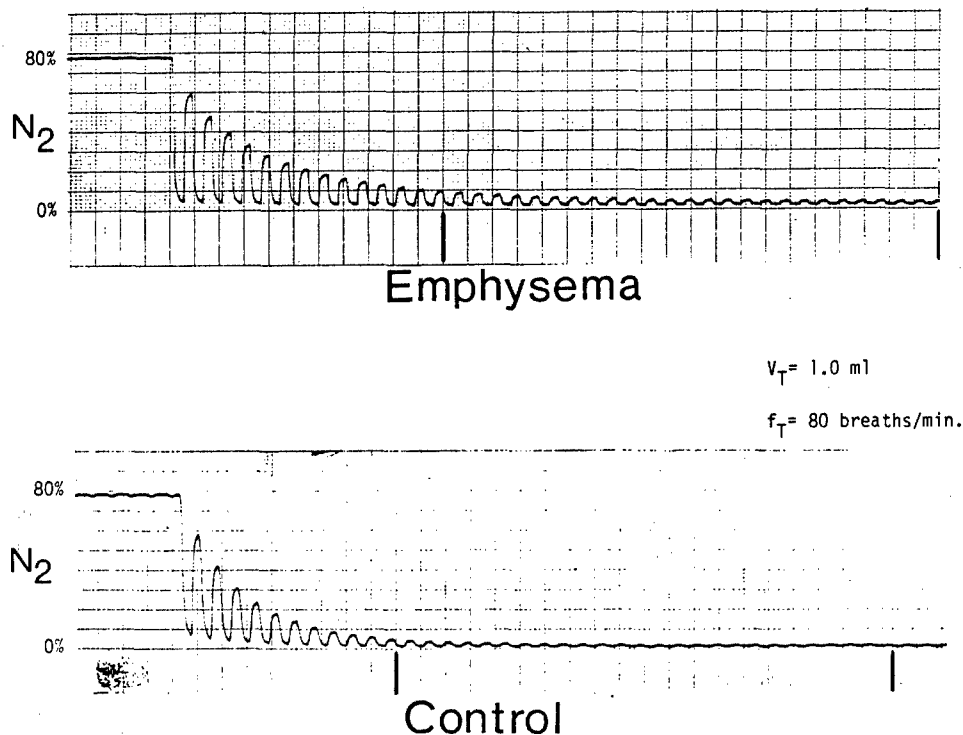


Figure 1. Airway nitrogen concentration from golden syrian hamsters ventilated with 100% oxygen. Emphysema was produced by the intratracheal instillation of 24 units of porcine pancreatic elastase/100 g body weight.

#### MAXIMAL EXPIRATORY FLOW-VOLUME (MEFV) RELATIONSHIPS

The MEFV is useful because it provides information on the functional status of airways, especially the small airways. Very fast measuring and recording equipment with demonstrated frequency response is required to successfully measure rapidly occurring maneuvers such as the MEFV. For use with small animals, flow plethysmographs which utilize stainless steel mesh screen as resistance elements have proven accurate and useful (Diamond and O'Donnell, 1977; Lucey et al., 1978; Mauderly et al., 1979; Sinnett et al., 1981). To measure a maximal expiratory flow-volume effort, the airway cannula of the experimental animal is exposed to a forcing function (that is a pressure sink maintained at 30-60 cm H<sub>2</sub>O less than  $P_B$ ). This requires very low impedance tubing (short with wide bore) and a low impedance valving system that permits production of a virtual step pressure change. Tests can also be accomplished using gases of different densities (e.g., helium-oxygen mixtures) which permit further definition of the diseased airways. Measurement of MEFV in small animals has been reported by Diamond and O'Donnell (1977), by Lucey et al. (1978), and by Sinnett et al. (1981) using flow plethysmographs.

## SINGLE BREATH OXYGEN TEST (CLOSING VOLUME)

Similar equipment to that used for the multiple breath nitrogen washout is required to accomplish this test. It is accomplished by inflating the animal to TLC with 100% oxygen and monitoring the airway N<sub>2</sub> concentration during a slow deflation maneuver. During the exhalation maneuver, the N<sub>2</sub> concentration initially rises quickly as the anatomical dead space is washed out. Then during the "alveolar plateau", the N<sub>2</sub> concentration rises more slowly as more distal gas exchange units contribute their gas. Finally, a rather sharp increase in N<sub>2</sub> concentration occurs near RV. The volume at which this increase occurs is called the closing volume and represents the point at which small airways actually start to collapse. The techniques to make these measurements have been described by Likens and Mauderly (1980) and by Loscutoff<sup>2</sup>.

## OTHER TESTS

There are other tests of pulmonary function which can be used in small animals. Airway response in bronchoprovocation can be measured in unanesthetized guinea pigs using the techniques for measurement of dynamic compliance and airway resistance which was described by Amdur and Mead (1958). New techniques to measure respiratory impedance in small animals are being developed by Jackson and his colleagues and have been reported in a separate paper in this symposium. With the development of equipment capable of measuring blood gases with very small samples, O'Brien and his co-workers (1979) have reported on the measurement of blood gases in unanesthetized hamsters at rest and during exercise (O'Brien et al., 1979). Such techniques will be useful to measure the shunt effect or to study gas exchange or respiratory control. Because of the current need and interest in this field, other new tests will be appearing which should be tried and incorporated into other tests already in use.

## EFFECTS OF EXPERIMENTAL EMPHYSEMA ON PULMONARY FUNCTION

Experimentally produced pulmonary emphysema is a useful model of pulmonary disease that is well documented and reported in the literature (Karlinsky and Snider, 1978; Kuhn and Tavossoli, 1976). For the studies reported here, emphysema was produced in golden syrian hamsters by the intratracheal instillation of 24 units of porcine pancreatic elastase per 100 g body weight (in 0.5 ml saline). The animals were held for three weeks before being studied. Lungs were fixed at 20 cm H<sub>2</sub>O with 2% glutaraldehyde infused via a low resistance fixation system (Hayatdavoudi et al., 1980) and the presence of emphysema was confirmed in tissue sections examined histologically.

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<sup>2</sup>Susan M. Loscutoff, Ph.D., Battelle-Northwest Pacific Lab, Richland, WA 99352.

Measurable changes in the pulmonary function have been found using the tests described above. Table 1 lists the changes in the mean linear intercept, lung volumes,  $D_{LCO}$ , and the multiple breath nitrogen washout in our hamsters. Snider and his co-workers (1977) have previously reported on changes in lung volumes and compliance of the lungs and chest wall. The changes observed in these two studies are parallel and complementary.

TABLE 1. PULMONARY FUNCTION CHANGES OF HAMSTERS WITH EXPERIMENTAL EMPHYSEMA (MEAN  $\pm$  SD)

	Normal	Emphysema
Body weight, g	155 $\pm$ 22	144 $\pm$ 23
Lung weight, g	0.77 $\pm$ 0.05	0.81 $\pm$ .23
Mean linear intercept, $\mu$ M	3.0 $\pm$ 9.8	1.0 $\pm$ 28.7
Total lung capacity, ml	7.6 $\pm$ 1.3	12.5 $\pm$ 2.8
Residual volume, ml	1.1 $\pm$ 0.6	2.6 $\pm$ 1.6
Diffusing capacity ( $D_{LCO}$ ), ml/min $\times$ torr <sup>-1</sup>	0.236 $\pm$ 0.069	0.094 $\pm$ 0.020
N <sub>2</sub> slope, log % breath <sup>-1</sup>	0.448 $\pm$ 0.068	0.226 $\pm$ 0.049
Breath index, # of breaths	9.0 $\pm$ 1.0	19.0 $\pm$ 2.0

The multiple breath nitrogen washout from a normal and an emphysematous hamster is shown in Figure 1. Although the interpretation of these changes is not altogether straightforward, it is clear that differences exist in these measurements which may be useful in estimating functional changes in the presence of pulmonary diseases.

Mauderly et al. (1979) have measured the difference in flow-volume relationships in normal vs. emphysematous rats and have found that flows were lower at all lung volumes in the diseased animals when compared to normal rats. The single breath oxygen test has been accomplished in rats by Lilkens and Mauderly (1980) who found that although the slope of phase III (the "alveolar plateau") was not different, the closing volume was shifted to a larger volume in the emphysemic as compared to the control animals.

#### FUNCTIONAL RESPONSE TO OTHER OXIDANT GASES

Following continuous exposure to 60% oxygen for 7 days, the TLC of rats was reduced by about 13% with no change in the  $D_{LCO}$  (when expressed as  $D_{LCO}/TLC$ ). Table 2 summarizes this information. In a separate study, hamsters were exposed for 21 days (23 h/d) to the reaction products of a complex mixture of ozone - sulfur dioxide - trans-2-butene. Following this exposure, we found that the  $D_{LCO}$  was increased significantly in both untreated

hamsters and in hamsters with experimental emphysema. Some of these data are shown in Table 3.

TABLE 2. DIFFUSING CAPACITY AND LUNG VOLUMES OF RATS FOLLOWING EXPOSURE TO 60% O<sub>2</sub> FOR 7 DAYS

	Control	60% O <sub>2</sub> exposed
Body weight, g	347 ± 20	357 ± 29
Lung weight, g	1.5 ± 0.2	1.5 ± 0.2
Total lung capacity, ml	14.4 ± 2.0	12.6 ± 2.0*
Diffusing capacity (D <sub>LCO</sub> ), ml/min x torr <sup>-1</sup>	0.206 ± 0.044	0.181 ± 0.035
D <sub>LCO</sub> /TLC	0.0147 ± 0.0043	0.0148 ± 0.0042

\*p < 0.05

TABLE 3. PULMONARY FUNCTION OF HAMSTERS WITH EXPERIMENTAL EMPHYSEMA EXPOSED FOR 21 DAYS TO A COMPLEX MIXTURE OF POLLUTANTS

	n	Air	Complex mixture
TLC, ml <sup>a</sup>	10	10.7 ± 0.9	10.3 ± 0.8
RV, ml	10	4.7 ± 0.7	5.1 ± 0.6
D <sub>LCO</sub> , ml/min x torr <sup>-1</sup>	10	0.088 ± .008	0.113 ± 0.007*
Lung weight, g	8	0.75 ± 0.01	0.77 ± 0.03
Breath index, # of breaths	6	12.1 ± 2.4	12.8 ± 1.9

<sup>a</sup>Adjusted means (±SE) by analysis of covariance.

\*Significantly different (p < 0.05) using Dunnett's Test.

### CONCLUSION

Useful tests of pulmonary function have been developed in recent years which can be utilized with small laboratory animals. These tests can be used to determine if pulmonary disease is present. In addition, the severity of such disease can be evaluated. The use of pulmonary function tests on animals following the inhalation exposure to environmental pollutants will provide valuable information regarding the health effects of such pulmonary insults.

Dose-response relationships could be determined for agents known to have pulmonary effects. Because these tests are adaptations of similar tests utilized in humans, extrapolation models may be developed which will help in the evaluation of the potential health effects of inhaled agents to which humans might be exposed.

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## OSCILLATORY MECHANICS IN NORMAL RAT LUNGS

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There are numerous advantages associated with using small laboratory animals, such as the rat, for physiological and toxicological studies. Methods for assessing pulmonary function in mammals are often ones originally devised for use in humans. However, application of these methods to study small mammals is often difficult because of their small size. Dynamic pulmonary function tests, used to detect alterations in airway caliber, that have been used in small mammals include measures of trans-pulmonary resistance during normal breathing (Amdur and Head, 1958), forced oscillatory resistance at a single frequency (Diamond et al., 1975), maximum forced expiratory flow-volume curves (Diamond and O'Donnell, 1977; Vinegar et al., 1979), and frequency dependence of compliance (D'Angelo, 1976; Diamond and O'Donnell, 1977). Recent studies performed in humans indicate that the most sensitive tests for small airways obstruction are frequency dependence of compliance and frequency dependence of resistance (Kjeldgaard, 1976). There are, however, methodological reasons why frequency dependence of resistance is a simpler test to employ.

This report describes a method for measuring the total respiratory system impedance in small mammals. From these data one could assess both frequency dependence of resistance and frequency dependence of compliance. Measurements on 31 normal rats indicate that the respiratory system of the rat behaves generally like a series mechanical network consisting of a resistive, compliant, and inertive element. This is behavior similar to that of the normal human. Parameter optimization techniques were used to derive resistance, compliance, and inertances of an equivalent network for each animal studied.

### METHODS

#### THEORETICAL CONSIDERATIONS

To avoid the frequency-response problem associated with the use of pneumotachometers for oscillatory flow measurements (Finucane et al., 1972; Jackson and Vinegar, 1979), we used a newly designed device (Jackson and Vinegar, 1979) that functions not only as the oscillatory flow generator but

also as the detector by which flow is measured. The system consists of a loudspeaker mounted between an airtight reference chamber in which a measure of pressure provides an estimate of speaker motion and a test chamber to which is attached the animal to be studied (Figure 1). The flow generated by the loudspeaker is measured by the ratio of pressure in the reference chamber,  $P_1$ , to the impedance of that chamber,  $-1/j\omega C_1$ , where  $j = \sqrt{-1}$ ,  $\omega = 2\pi f$  with  $f$  the frequency of oscillation, and  $C_1$  = the compliance of the reference chamber. That same flow, if connection T is intact, equals the sum of flows through the two parallel pathways, each given by the ratio of pressure in the test chamber,  $P_2$ , to the respective impedances,  $Z_2$  and  $Z_{rs}$ :

$$-j\omega C_1 P_1 = P_2/Z_2 + P_2/Z_{rs}. \quad (1)$$

Equation (1) can be solved in terms of the oscillatory flow into the animal,  $\dot{V}_{os} = P_2/Z_{rs}$ , or:

$$\dot{V}_{os} = j\omega C_1 P_1 + P_2/Z_2. \quad (2)$$

Dividing both sides of equation (2) by  $P_2$  and reordering, we obtain the following expression for the respiratory system impedance:

$$Z_{rs} = 1/(j\omega C_1 P_1/P_2 - 1/Z_2) \quad (3)$$

Thus, provided that  $C_1$  and  $Z_2$  are known, we can determine flow through the load as well as the animal's respiratory system impedance from measurements of pressure in the reference and test chambers.

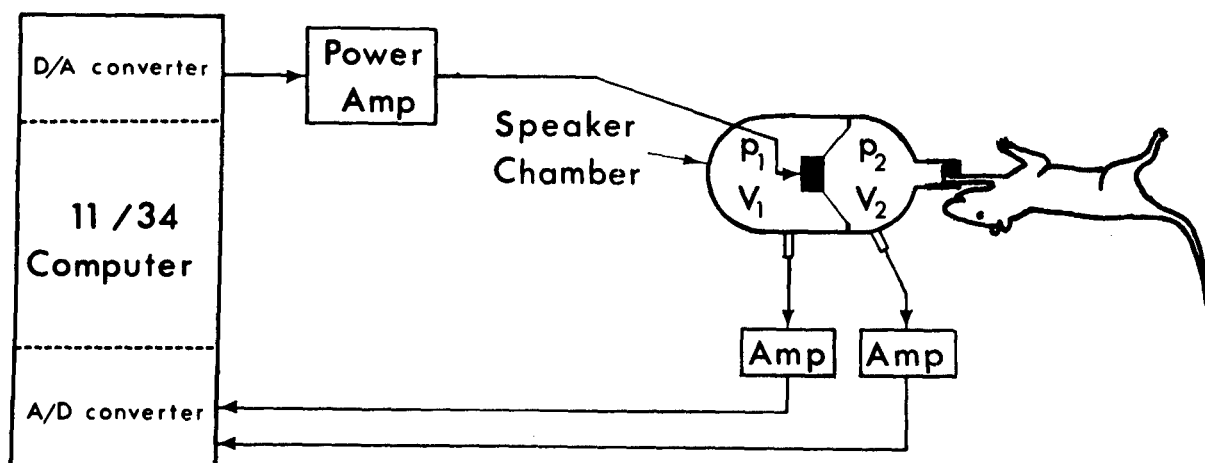


Figure 1. Schematic diagram of apparatus used to measure mechanical impedances.

The compliance of the reference chamber,  $C_1$ , is given by:

$$C_1 = V_1 / (P_{\text{atm}} \gamma) \quad (4)$$

where  $V_1$  is the volume of the chamber in liters,  $P_{\text{atm}}$  the barometric pressure in cm H<sub>2</sub>O, and  $\gamma = 1.4$ , the ratio of specific heats at constant pressure and constant volume for adiabatic compression in air. We measured  $V_1$  by water displacement. The volume of the test chamber is difficult to measure because of its irregular geometry. We therefore preferred to determine  $Z_2$  empirically from measurements of  $P_1$  and  $P_2$  obtained with the connection T interrupted so that flow through the port equals zero, and equation (1) becomes:

$$Z_2 = -P_2 / P_1 j \omega C_1 \quad (5)$$

### EQUIPMENT

A standard loudspeaker (901 series 2, Bose, Inc., Framingham, MA) is mounted in a cylinder with hemispherical domes at either end. The domes and cylinder are made of 0.635-cm thick Lucite<sup>tm</sup>. The speaker divides the cylinder into two asymmetrical chambers. The volume of the reference chamber (#1 in Figure 2) is approximately four times larger than that of the test chamber (#2 in Figure 2). A pressure transducer whose frequency-response was determined by us to be uniform from DC to at least 300 Hz (model 237, Setra, Inc., Natick, MA) is mounted in the wall of each chamber.

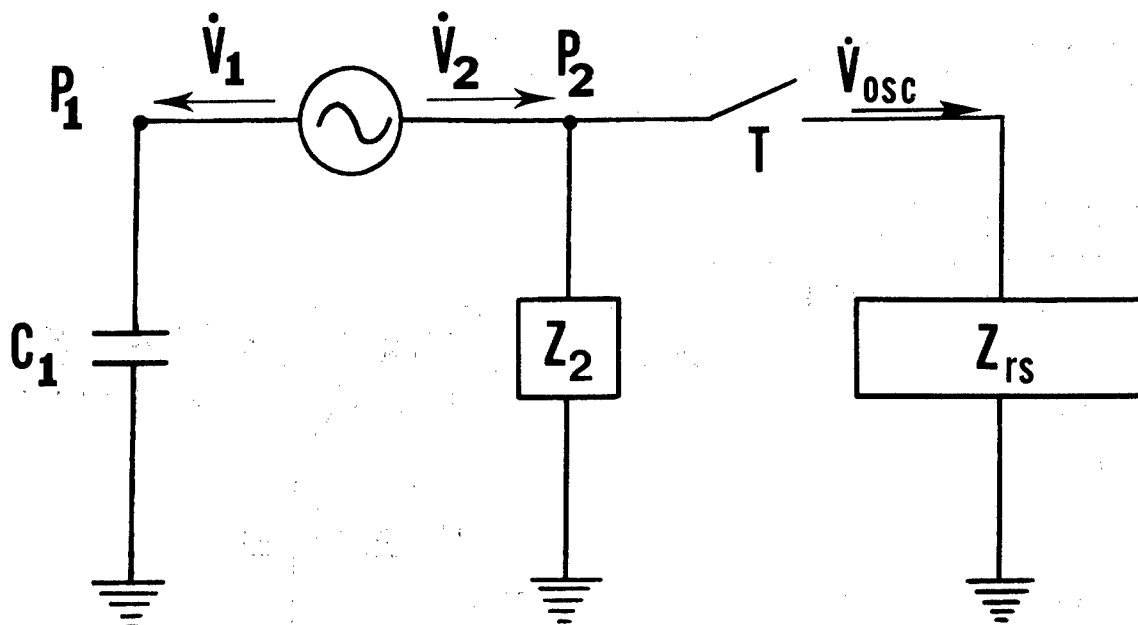


Figure 2. Analogous electrical network of speaker chamber in Figure 1. See text for definition of variables and parameters.

Sinusoidal functions are generated by a digital computer (PDP 11/34, Digital Equipment Corp., Maynard, MA). After digital-to-analogue conversion (D/A Model AA-11K, DEC) and amplification (model D150A, Crown, Elkhart, IN), the oscillatory signal is applied to the loudspeaker. The computer program includes a feedback loop that adjusts the amplitude of the D/A output so that peak oscillatory flows are the same at all frequencies. During oscillatory measurements,  $P_1$  and  $P_2$  are separately amplified and, with a filter attenuation of 24 db/octave, filtered below 5 Hz and above 200 Hz (models PS-11, 451, and 4113, Itacho, Ithaca, NY).  $P_1$  and  $P_2$  are then converted from analogue to digital form (A/D, model AD-11K, DEC) for subsequent analysis.

## DATA ANALYSIS

The signals  $P_1$  and  $P_2$  are of the form:

$$P_i(t) = A_i \sin(\omega t) + B_i \cos(\omega t) \quad (6)$$

where  $i = 1$  or  $2$ . The sine component equals the real or in-phase portion and the cosine component the imaginary or out-of-phase portion of each signal. The coefficients of the sine and cosine components of such signals can be computed by the relationships:

$$A_i = (1/\pi) \int_0^{2\pi} \sin(\omega t) P_i(t) dt \quad (7)$$

and

$$B_i = (1/\pi) \int_0^{2\pi} \cos(\omega t) P_i(t) dt \quad (8)$$

Discrete forms of (7) and (8) are given by:

$$A_i = (2/N) \sum_{n=1}^N \sin(2n\pi/N) P_i(n) \Delta t \quad (9)$$

and

$$B_i = (2/N) \sum_{n=1}^N \cos(2n\pi/N) P_i(n) \Delta t \quad (10)$$

where  $N$  is the total number of samples per cycle with period of  $N\Delta t$ .

At each frequency, 12 cycles were generated. The response of the first 4 cycles was ignored because of possible transients from frequency increments. During the next 8 cycles, each input signal  $P_1$  and  $P_2$  was sampled at 10 equally spaced times per cycle ( $N = 10$  in eqn. 9 and 10). The 8 sets of 10

data points acquired by each channel at each frequency were then ensemble averaged. Frequency was incremented in discrete 2-Hz steps from 20 to 128 Hz. The sine and cosine components of each signal were estimated by using equations (9) and (10), with  $P_i(n)$  the 10-point ensemble average for each channel ( $n = 1, 10$ ). Pressures  $P_1$  and  $P_2$  were converted to complex numbers, and the complex ratio  $P_2/P_1$  was used to solve equations (2) and (3).

## CALIBRATIONS

To match transducer outputs, both transducers were temporarily mounted in the test chamber. Pressure outputs  $P_1$  and  $P_2$  were compared at all frequencies that were to be ultimately used. Differences in phase and amplitude of  $P_2$  with respect to  $P_1$  at each frequency were stored and used to correct all subsequent  $P_2$  signals.

To determine the test chamber impedance,  $Z_2$ , the port in the test chamber was closed and a frequency sweep generated.  $Z_2$  was then computed (eqn. 5), stored for each frequency, and later used to calculate oscillatory flow and impedance with equations (3) and (5).

Absolute pressure calibrations were not required for measuring oscillatory resistance, because in the calculation of  $Z_{RS}$  (eqn. 3), pressures appear only as the ratio  $P_1/P_2$ . Scaling for the impedance measurements was given by the volume of the reference chamber, as it appears in the expression for  $C_1$ .

## SELECTION OF SYSTEM PARAMETERS

We used a scheme described by Peslin et al. (1975) to select the parameters of a lumped mechanical network whose response compared best to the experimental data. Briefly, this technique is accomplished by selecting a network that seems realistic, picking values for the various elements within the network, then comparing the impedance of the model to the experimental data. New estimates for the elements are chosen, and the impedance of the model with these parameter values is compared to the impedance of the previous model. If these new parameters improve the comparison between the model's impedance and the data, the search continues. By this iterative process parameter values are selected so that the differences between the model's impedance and the data are minimized.

To compare the impedance of the model to that of the data, it is necessary to select some quantitative parameter that is representative of these differences. We used the average distance ( $D$ ) between the computed real and imaginary parts of the impedance of the model and the data, that is given by:

$$D(\%) = \frac{100}{n} \sum_{i=1}^n \frac{(R_c - R_d)^2 + (X_c - X_d)^2}{R_d^2 + X_d^2} \quad (11)$$

where R is resistance (real part of impedance), X is reactance (imaginary part of impedance), the subscript c is used to represent the computed model parameters, and the subscript d is used to represent the measured respiratory system parameters. This difference, D, is minimized by an iterative technique on a DEC 11/34 computer. The resulting minimum D is called the residual difference,  $D_r$ . The magnitude of  $D_r$  depends upon the values of the model's parameters as well as upon experimental factors like noise and the distribution of the data points with respect to the independent variable, frequency. Thus,  $D_r$  should be used only to compare the agreement between the data and various models.

## EXPERIMENTAL PROTOCOL

Studies were carried out on 31 male Sprague-Dawley, CRD-free rats weighing between 254 and 482 grams. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (45 mg/kg). The trachea was cannulated in the cervical region with a blunted needle cannula (12 gauge, stainless steel, luer fitting, 3.5 cm in length) that was passed through a rubber stopper for connection to the port of the test chamber of the high frequency oscillator. The tip of the cannula was 1.5 cm below the larynx. All the rats were studied in the supine position. During the test the rats breathed room air from the sealed oscillating chamber. Between runs the rats were disconnected from the oscillating chamber while it was flushed with room air.

Impedances of the tracheal cannula alone were measured at each of the frequencies used. Resistances and reactances were stored, and these values were subtracted from all subsequent measurements, which included the animal plus the tracheal cannula. Thus, all impedance values reported here represent impedances of the animal only. The tracheal cannula behaves like a resistor and inertial element in series. With the flow amplitudes and frequencies used, mean resistance of the tracheal cannula was 20 cm H<sub>2</sub>O/l/sec while its inertance was .106 cm H<sub>2</sub>O/l/sec<sup>2</sup>.

## RESULTS AND DISCUSSION

DuBois et al. (1956), who made the first impedance measurements in normal humans, found that resistance was nearly constant over the frequency range tested (2-15 Hz) and that reactance was negative at low frequencies, became equal to zero (at approximately 6 Hz), then became positive at higher frequencies. We found that in some normal rats, respiratory system impedances (Figure 3a) had frequency-dependent behavior similar to man, except the resonant frequency (i.e., the frequency where reactance equals zero) occurred at much higher frequencies. Frequency-dependent behavior such as this is typical of a mechanical system consisting of a single resistance (R), a single compliant element (C), and a single inertial element (L), all connected in series. As a first approximation we used this mechanical model (i.e., a series RLC network) in our parameter optimization scheme to estimate values of R, C, and L whose impedance was the optimum fit of the experimental data. The resulting optimized parameters for each of the animals studied are given in Table I.

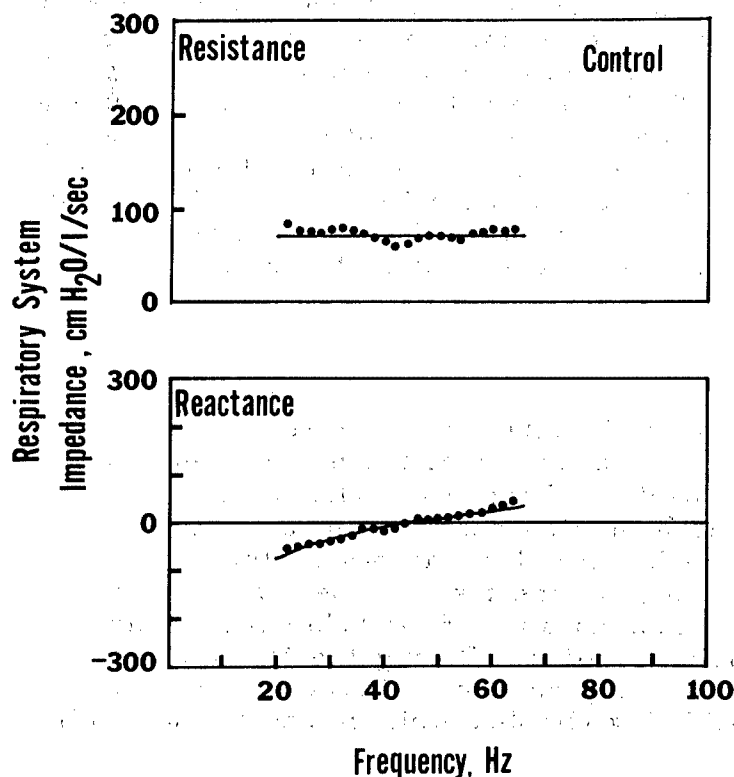


Figure 3a. Respiratory system impedances of rat #1 (Table I), shown as resistance, or real part of impedance (upper panels), and reactance, or imaginary part of impedance (lower panels).

TABLE I. OPTIMIZED PARAMETERS NORMAL RATS

Rat #	Weight (grams)	R (cm H <sub>2</sub> O/l/sec)	C (l/cm H <sub>2</sub> O X 10 <sup>-4</sup> )	I (cm H <sub>2</sub> O/l/sec <sup>2</sup> )	$\omega_0$ (Hz)	D <sub>r</sub>
1	254	71	1.87	.154	43	13
2	318	88	1.05	.177	37	12
3	330	182	1.03	.144	41	15
4	330	111	1.38	.158	34	19
5	332	198	1.24	.137	38	17
6	335	112	1.14	.138	40	12
7	336	147	1.48	.084	45	22
8	337	101	1.23	.152	37	13
9	346	70	1.24	.142	38	13
10	350	84	1.10	.133	42	13
11	353	123	1.29	.129	39	22
12	353	90	1.20	.113	43	16
13	366	73	1.50	.122	37	21
14	370	89	1.09	.113	45	17
15	372	112	1.43	.083	46	12
16	388	75	1.43	.101	42	26
17	400	100	1.07	.108	47	25
18	401	107	1.22	.103	45	21
19	402	100	1.31	.103	43	19
20	403	90	1.50	.123	37	15
21	410	151	1.70	.040	61	18
22	412	72	1.80	.043	57	31
23	412	88	1.43	.099	42	23
24	421	107	1.47	.070	50	17
25	426	78	1.21	.128	40	20
26	427	103	1.20	.138	39	19
27	428	90	1.10	.175	36	14
28	446	85	1.46	.141	35	16
29	452	101	1.46	.128	37	22
30	469	99	1.61	.106	39	19
31	482	98	2.14	.120	31	19
Mean	382	103	1.33	.120	42	
SD	50	30	.26	.033	6.2	
Coef. of var.	13%	29%	19%	27%	15%	

Mean respiratory system resistance in the animals studied here (103 cm H<sub>2</sub>O/l/sec) was less than half the value for pulmonary resistance reported by Diamond and O'Donnell (1977) (260 cm H<sub>2</sub>O/l/sec). This difference could possibly be due to the resistance of the tracheal cannulas since Diamond and O'Donnell did not subtract the resistance of their cannulas as we did. The resistance of our cannula was only 20 cm H<sub>2</sub>O/l/sec, which does not account for the differences between our respective resistances, but if a smaller diameter and/or longer tracheal cannula was used by Diamond and O'Donnell it is possible that its resistance could be of sufficient magnitude to explain this difference. Our mean compliance ( $1.33 \times 10^{-4}$  l/cm H<sub>2</sub>O) does compare favorably with dynamic compliance reported by Diamond and O'Donnell (1977) ( $2.0 \times 10^{-4}$  l/cm H<sub>2</sub>O). To our knowledge no other investigators have measured inertance in rats to which we can compare our values. Resonant frequencies in guinea pigs have, however, been found to be about 32 Hz (Hiatt, 1974), which compares favorably with resonant frequency in the rat as measured by us (42 Hz).

In not all instances did the impedances have frequency-dependent behavior like that of the rat in Figure 3a. Several had frequency-dependent behavior in resistance where resistance increased with increasing frequency (Figure 3b). Frequency dependence of resistance such as was seen in this rat can be due to: 1) nonlinear behavior of airway resistance caused by distortions in their velocity profiles (Finucane et al., 1975), or; 2) inhomogeneities in the RC time constants or RL frequency constants of parallel airway pathways. The degree to which velocity profile distortions produce frequency dependence of resistance can be predicted on a theoretical basis. Crandall (1926) developed equations describing the frequency-dependent behavior of oscillatory resistances in straight, unbranched tubes. Even though these equations were developed for describing flow of incompressible fluids, it has recently been shown that at these frequencies they are also applicable to compressible fluids (Dorkin et al., 1980). Crandall's work indicated that the degree to which resistance is affected by velocity profile distortions is conveniently expressed in terms of the dimensionless parameter  $\alpha$ , where  $\alpha = r\sqrt{2\pi f/\nu}$  ( $r$  = tube radius,  $f$  = frequency in Hz, and  $\nu$  = dynamic viscosity of the fluid) and that resistance begins to increase with frequency when  $\alpha > 1$ . Note that velocity profile distortion at a given frequency is most severe in airways with the largest radius, in this case the trachea. Assuming that tracheal size in these rats was approximately equal to the outer diameter of the tracheal cannula (12 gauge, or  $r = .02$  cm), one finds that  $\alpha = 1$  at a frequency of about 50 Hz. Since this frequency is within the frequencies used, it is possible that some of the frequency-dependent behavior in resistance is due to this phenomenon. In addition, we found generally that the degree of frequency dependence of resistance increased with the size of the rat, and thus presumably with the size of the trachea. This is consistent with the above hypothesis that frequency dependence of resistance is, at least in part, due to velocity profile distortions.

The reactance of the RLC series model consistently compared well with the experimental reactances. This is evidence that frequency dependence of compliance (at low frequencies) does not occur in the normal rat.



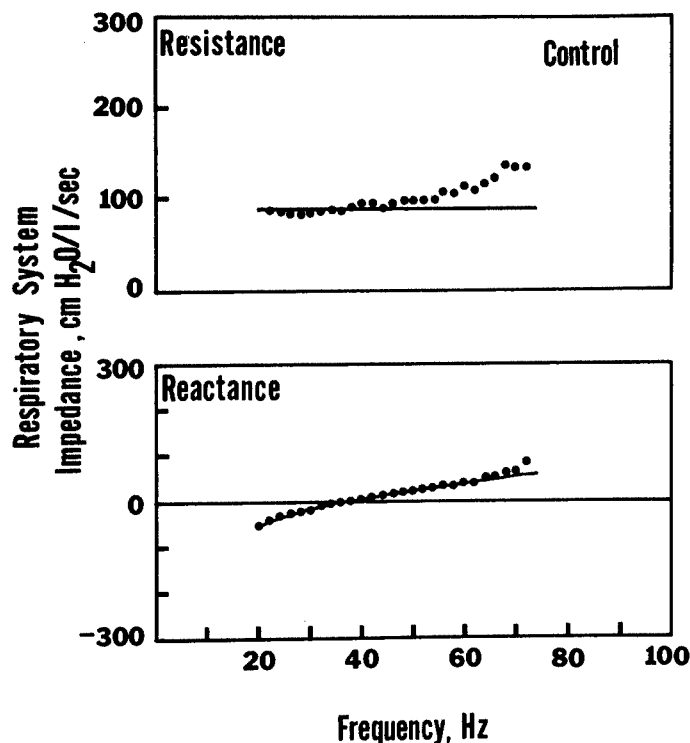


Figure 3b. Respiratory system impedances of rat #27 (Table I).

We have not yet investigated the use of more complex models (i.e., ones including RLC pathways, or ones including airway wall properties) to describe rat impedances. It is possible that impedances of these models will better fit the data since their resistances can be frequency dependent. Use of more complex models could also provide more information about the lung under normal and diseased conditions. As an example, using a model incorporating airway wall properties may provide evidence of the distribution of resistance between central and peripheral airways. These techniques have successfully been used in dogs (Pimmel et al., 1978) and humans (Williams et al., 1979).

To summarize, we have described a technique for measuring respiratory system impedances in small mammals and have presented measurements in normal rats. The mechanical behavior of the normal rat lung is similar to that of the normal human lung in that it behaves much like a series RLC network. Also, like the human, resistance is frequency dependent in some cases, and this appears to be due to nonlinear behavior of resistance within the larger airways.

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#### ACKNOWLEDGEMENTS

This work was supported by U. S. Environmental Protection Agency Grant 807661-01 and a contract from the Electric Power Research Institute (RP 1112-2).

BIOCHEMICAL CORRELATES OF AIRWAY AND TISSUE INJURY IN  
RODENTS EXPOSED TO NO<sub>2</sub> \*

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INTRODUCTION

The analysis of body fluids such as blood and urine has long been used in the detection of pathologic conditions of the body. We have been investigating the efficacy of analysis of airway fluids, sampled by pulmonary lavage or by segmental washings, as a method for detecting lung injury in experimental animals exposed to potential pollutants (Henderson et al., 1978; Henderson et al., 1978; Henderson et al., 1979; Henderson et al., 1979). Such a method would be useful as a rapid screening tool to determine which compound in a series of compounds was the most toxic to the lung and at which level of exposure it was toxic. Earlier investigations showed that the inflammatory response preceding the acute, diffuse lung injury caused by inhalation of cadmium chloride could be easily detected by biochemical and cytologic changes in lung lavage fluids (Henderson et al., 1978). The present study was designed to determine; a) the best indicators of damage which can be detected in lavage fluid from animals with a multifocal-type pulmonary injury such as the terminal bronchiolitis resulting from an inhaled oxidant; b) the lowest level of lung injury that can be detected by assay of the parameters in our test system, and; c) the usefulness of measurement of protease levels in lavage fluid or lung tissue as indicators of the early stages of developing chronic, emphysematous-like lesions. To achieve the first two objectives, we examined the enzymatic activity and cytologic composition of lung washings from Syrian hamsters exposed to graded levels of NO<sub>2</sub> for 48 hours. We also looked at enzymatic levels in whole lung washings and lung tissue homogenates from Fischer 344 rats exposed for up to eight weeks to 20 ppm NO<sub>2</sub>.

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\* Research supported in part by the EPA via Interagency Agreement EPA-IAG-D5-E61 under U. S. DOE Contract Number DE-ACO4-76EV01013.

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## MATERIALS AND METHODS

Three groups of 20 male and 20 female adult (12-16 week old) Syrian hamsters [Sch:(SYR)] each were exposed to atmospheres containing mean concentrations of  $12.0 \pm 0.6$ ,  $16.7 \pm 0.5$  and  $22.4 \pm 1.5$  ppm ( $\pm$  SD) NO<sub>2</sub> gas, respectively, for 48 continuous hours. The NO<sub>2</sub> gas was generated from nitrogen tetroxide and exposures were conducted using 27 inch, stainless steel, Laskin type exposure chambers. The NO<sub>2</sub> concentrations were monitored continuously with a chemiluminescent NO<sub>2</sub> analyzer as well as periodically by the Saltzman technique (Saltzman, 1949-1955). Temperature levels during exposure were 70 to 80°F and relative humidity was approximately 40%. One group of 24 male and 24 female adult hamsters were exposed to clean filtered air for 48 continuous hours in similar chambers and under similar environmental conditions to serve as controls.

At 1, 2, 8, and 21 days after initiation of the exposure, three male and three female hamsters from each group were killed via exsanguination while under 5% halothane anesthesia. The heart-lung blocks were removed and weighed and the lungs lavaged twice with 4.0 mL of 0.15 M saline. Recovered lavage fluid was collected in clean glass tubes and refrigerated (5°C). The right lungs were selectively perfused intravascularly with 0.15 M saline to remove peripheral blood.

The right lungs were homogenized in 2.0 mL of cold (5°C) 0.25 M sucrose containing 0.05 M Tris buffer, pH 7.4 in a Tissumizer® (Tekmar, Cincinnati, OH) for 30 seconds at 50,000 rpm. Homogenates were centrifuged at 300 X *g* for 10 minutes and the gauze-filtered supernatants kept at 5°C for biochemical analysis. Collected lavage fluids were centrifuged at 300 X *g* for 10 minutes and the supernatants kept at 5°C for biochemical analysis.

The cellular pellets resulting from lavage fluid centrifugation were resuspended in 1.0 mL of 0.15 M saline. A Coulter counter (Coulter Electronics, Inc., Hialeah, FL) was used to determine total erythrocyte and nucleated cell counts. Cells were transferred to glass slides by centrifugation at 700 rpm for 5 minutes with a Cytocentrifuge (Shandon Southern Instruments LTD, Canberry, Surrey, England). Differential cell counts were performed on all Wright-Giemsa or Harleco's Diff Quik® (Harleco, Gibbstown, NJ) stained cytocentrifuge samples.

Parameters measured in the cell-free portion of lavage fluids and tissue homogenates were selected for their potential to serve as indicators of pulmonary injury, repair and adaptation development (Henderson et al., 1979). These parameters are listed in Table 1 with their tissue or cellular specificity, cellular location, and possible indication if elevated. Total protein and sialic acid were measured only in lavage fluids. Assays for lactate dehydrogenase (LDH), glucose-6-phosphate-dehydrogenase (glu-6P-DH), glutathione reductase, alkaline phosphatase, acid phosphatase, and  $\beta$ -glucuronidase were made by the technique of Bergmeyer et al. (1974). Glutathione peroxidase was measured by the technique of Little et al. (1970). Total soluble protein levels were measured by the technique of Lowry (1951). Sialic acid levels were measured by the method of Downs and Pigman (1976). The LDH, glu-6P-DH, glutathione

reductase, alkaline phosphatase, and total soluble protein assays were adapted to an automated miniature centrifugal analyzer (Multistat III, Instrumentation Lab., Lexington, MA).

TABLE 1. BIOCHEMICAL PROFILE OF PULMONARY LAVAGE FLUIDS AND TISSUE HOMOGENATES

Biochemical Parameter	Cellular Location	Possible Indication
Lactate Dehydrogenase	Cytosol (glycolysis)	Cell damage (Increased membrane permeability to frank cell lysis)
Glucose - 6 Phosphate-dehydrogenase	Cytosol (hexose mono-phosphate shunt)	1. Cell damage 2. Activation of macrophages
Acid Phosphatase and $\beta$ -glucuronidase	Cytosol (lysosomal enzymes)	1. Release during phagocytosis 2. PMN and/or macrophage damage
Alkaline Phosphatase	Plasma membranes	1. Cell membrane rupture 2. Transudation of serum proteins
Glutathione Peroxidase	Cytosol	Protection mechanism activated against lipoperoxidation
Glutathione Reductase	Cytosol	Regeneration mechanism for glutathione peroxidase system
Total Protein	Extracellular	Transudation of proteins across alveolar-capillary barrier
Sialic Acid	Cytosol and extra-cellular	Increased mucus secretion in response to irritation

Morphologic pulmonary alterations were evaluated at 1, 2, 8, and 21 days after initiation of exposure to  $\text{NO}_2$ . Four animals (two males and two females) from each exposure level and six control animals (three males and three females) were killed via exsanguination while under 5% halothane anesthesia on each day. Gross alterations noted at necropsy were recorded. The right lung lobes were perfused intratracheally with a 10% neutral buffered formalin solution. Tissues were embedded in paraffin, sectioned at 5 microns and stained with hematoxylin and eosin for light microscopic examination.

For studies on developing chronic lung pathology, Fischer 344 rats, which were laboratory reared and specific pathogen free, were exposed in four, 27-inch, Laskin type, whole-body exposure chambers in groups of 64 to  $\approx$  20 ppm NO<sub>2</sub> for 24 hours/day, five days/week for eight weeks or were sham exposed to filtered air. Chamber nitrogen dioxide concentrations were determined as described above. Mean NO<sub>2</sub> concentration (Saltzman) was  $20 \pm 2$  ppm for the eight-week exposures.

Six control and exposed rats (3 males and 3 females) were sacrificed at 2 days, 2 weeks, 2, 6½, and 12 months after exposure using halothane anesthesia and cervical dislocation. Lungs were lavaged and the right diaphragmatic lung lobe homogenized in 0.25 M sucrose in 0.05 M Tris buffer at pH 7.4. Biochemical constituents (LDH, alkaline phosphatase, protein) of lavage (airway) and homogenate (tissue) supernatant fluid and cellular composition of lavage fluid were noted (Henderson et al., 1979). Protease activity was quantitated as mg <sup>14</sup>C-leucine-globin released/hour/kg body weight and determined at pH 5.0, 7.2, and 8.0 at 25°C in the presence of 0.02 M cysteine to activate thiolproteinases (Barrett, 1980).

For histopathology studies, four exposed and four control rats (two male and two female) were sacrificed at the same time periods and the lungs endotracheally perfused for 24 hours with 10% neutral buffered formalin at 20 cm H<sub>2</sub>O pressure. Lungs were sectioned and stained with hematoxylin and eosin. The mean linear intercept (MLI) of alveolar walls was determined using the procedure of Dunhill (1962). Each measured parameter of the study, except mean linear intercept, was corrected for any difference in body mass by expressing it as the amount present in lung per kg body weight.

The Mann-Whitney U statistic was used to detect differences in central tendency for all tests in the study.

## RESULTS

The peak biochemical and cytologic response in the lavage fluid from the hamsters was seen at 2 days after initiation of the acute exposures (Figure 1). Protein levels in the lavage fluid were also elevated. While all the biochemical and cytologic parameters measured showed a dose-related response, by far the most sensitive indicator of the terminal bronchiolitis caused by NO<sub>2</sub> inhalation was the influx of neutrophils. The graded levels of injury caused by the exposures to different concentrations of NO<sub>2</sub> indicated an increasingly extensive necrotizing terminal bronchiolitis (Figure 2).

Biochemical analysis of the lung homogenates showed an increase in enzymatic activities as well as the total protein in the homogenates (Table 2). The latter is probably a good measure of the degree of cellular influx into the injured lung as well as the inability to adequately perfuse the vasculature of the injured lung. The only enzymatic activities that increased more than the protein content were alkaline phosphatase and glucose-6P-dehydrogenase.

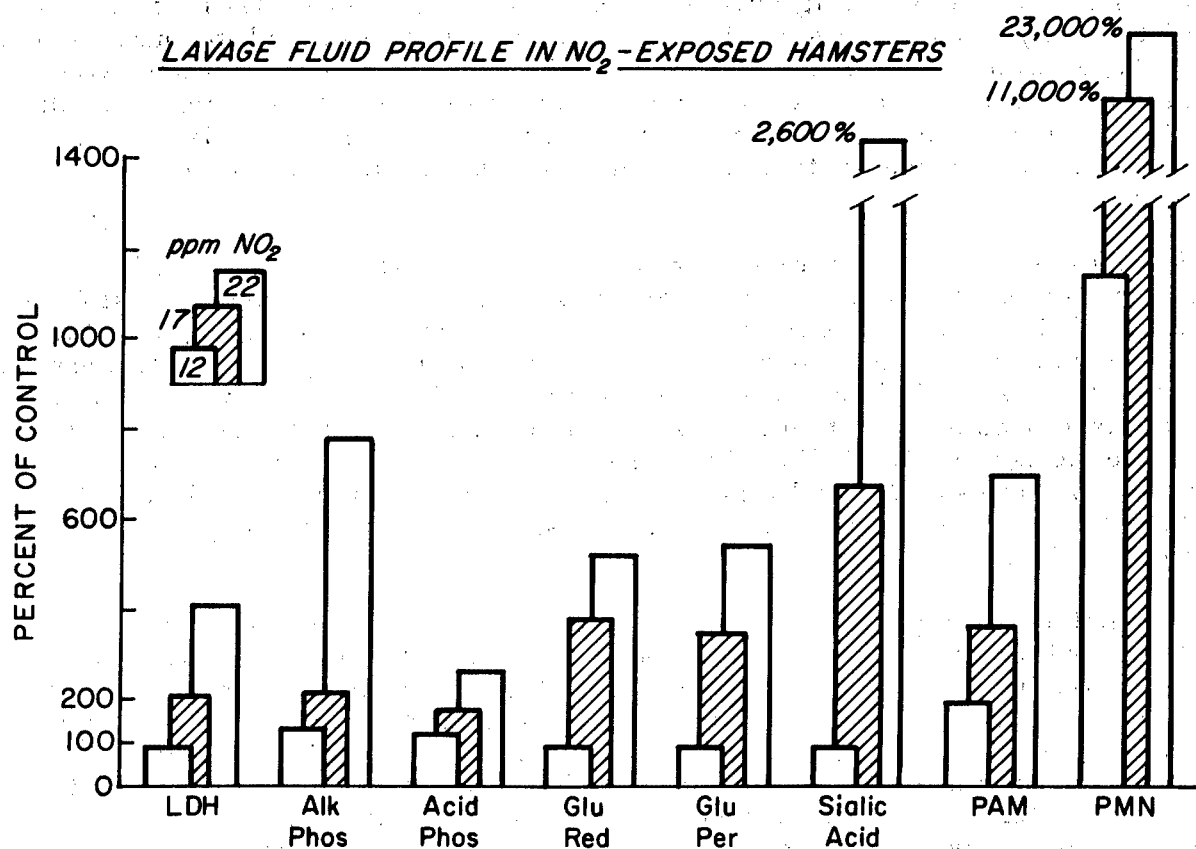


Figure 1. Levels of biochemical and cytologic indicators of pulmonary injury detected in lung washings from hamsters two days after initiation of exposure to 12, 17, or 22 ppm NO<sub>2</sub>. Each bar represents the mean of results from 6 animals. LDH = lactate dehydrogenase; alk phos = alkaline phosphatase; acid phos = acid phosphatase; glu red = glutathione reductase; glu per = glutathione peroxidase; PAM = pulmonary alveolar macrophages; PMN = polymorphonuclear leukocytes. Levels of the biochemical measurements are significantly different from controls ( $P \leq 0.05$ ) for the 17 and 22 ppm NO<sub>2</sub> exposures. Cytologic changes were significantly different from controls at all three exposure levels.



Figure 2a. Lungs from hamsters immediately after a 48-hour exposure to 0, 12, 17 or 22 ppm  $\text{NO}_2$ . Hematoxylin and eosin stain (224 x).

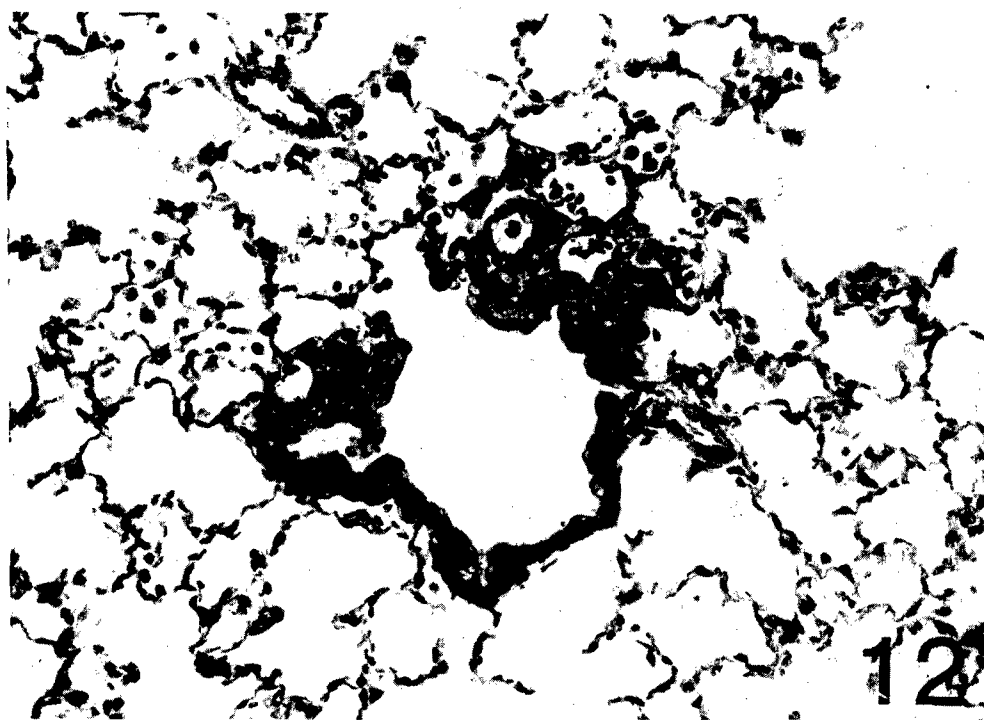


Figure 2b. Lungs from hamsters immediately after a 48-hour exposure to 0, 12, 17 or 22 ppm  $\text{NO}_2$ . Hematoxylin and eosin stain (224 x).



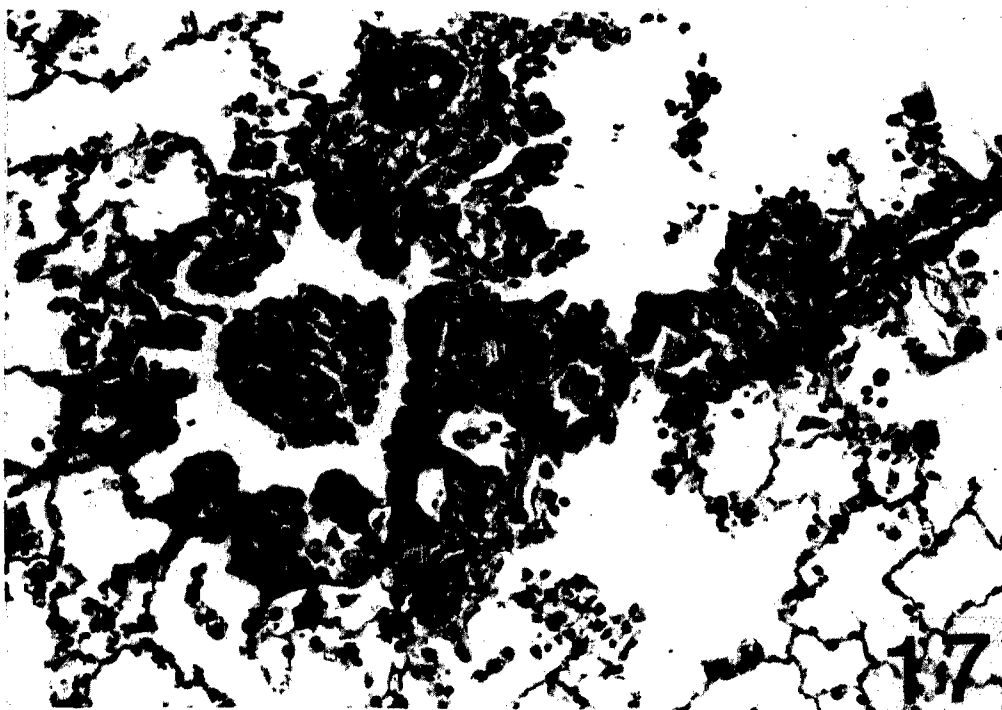


Figure 2c. Lungs from hamsters immediately after a 48-hour exposure to 0, 12, 17 or 22 ppm  $\text{NO}_2$ . Hematoxylin and eosin stain (224 x).

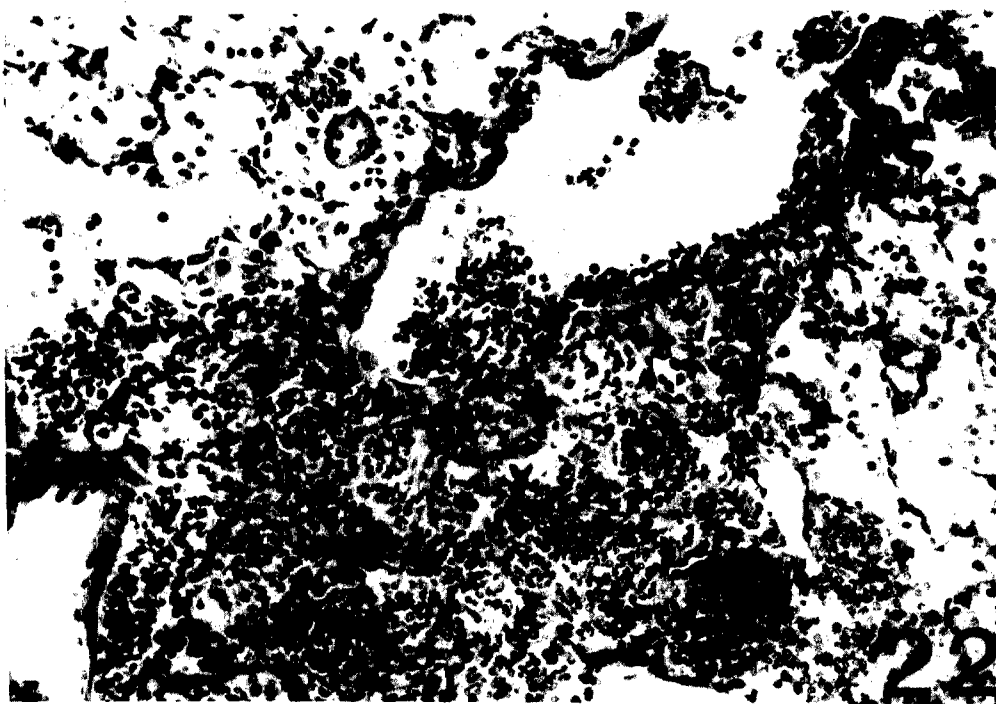


Figure 2d. Lungs from hamsters immediately after a 48-hour exposure to 0, 12, 17 or 22 ppm  $\text{NO}_2$ . Hematoxylin and eosin stain (224 x).

TABLE 2. BIOCHEMICAL RESPONSES IN PULMONARY TISSUES FROM SYRIAN HAMSTERS 2 DAYS AFTER INITIATION OF EXPOSURES TO VARIOUS NO<sub>2</sub> CONCENTRATIONS\*

Parameter	NO <sub>2</sub> Concentration (ppm)			
	0	12	17	22
LDH	260 ± 44	354 ± 92 <sup>†</sup>	348 ± 48 <sup>†</sup>	641 ± 101 <sup>†</sup>
Alkaline Phosphatase	2.5 ± 0.9	3.7 ± 1.6	7.3 ± 1.6 <sup>†</sup>	15.9 ± 5.8 <sup>†</sup>
Acid Phosphatase	7.1 ± 0.7	9.3 ± 2.2	9.1 ± 1.0 <sup>†</sup>	10.5 ± 0.9 <sup>†</sup>
Glutathione Reductase	16.5 ± 3.1	19.5 ± 3.5	23.2 ± 2.2 <sup>†</sup>	24.4 ± 3.8 <sup>†</sup>
Glutathione Peroxidase	8.5 ± 2.1	9.3 ± 1.0	11.0 ± 2.3 <sup>†</sup>	12.3 ± 2.0 <sup>†</sup>
Glu-6P-DH	8.1 ± 1.6	10.7 ± 3.4	16.7 ± 3.0 <sup>†</sup>	32.9 ± 6.2 <sup>†</sup>
β-Glucuronidase	0.9 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.4 ± 0.2 <sup>†</sup>
Total Protein	269 ± 34	297 ± 64	818 ± 255 <sup>†</sup>	625 ± 99 <sup>†</sup>

\* Values are means of six samples ± SD. All values are given as IU/lung/kg body weight (enzymes) or mg/lung/kg body weight (protein).

<sup>†</sup> Value differs significantly from control ( $p < 0.05$ ) by Mann-Whitney U Test.

In the study of rats exposed to 20 ppm NO<sub>2</sub> for up to eight weeks, animals observed at 2 days after initiation of the exposure had lavage fluid and tissue changes similar to those observed in hamsters in the previously described acute study. Histologically observed pulmonary edema was consistent with the ~ 10 fold increase in airway protein. Airway neutrophils (as sampled by lavage) were increased approximately 8 fold and a multifocal terminal bronchiolitis was present. Airway LDH and alkaline phosphatase were elevated (410% and 230% of control, respectively,  $p \leq 0.05$ ).

By 14 days, the lavage fluid neutrophil content had returned to normal but the pulmonary macrophage count was elevated approximately 3 fold. The LDH and alkaline phosphatase activity remained above controls but decreased from the 2 day levels (280% and 190% of control level, respectively,  $p < 0.05$ ). Alveolar walls were slightly thickened by apparent interstitial cell proliferation. Increased volume of lungs (150% of control, corrected for differences in body mass,  $p \leq 0.05$ ) was present and remained for 6 months after initiation of the exposure.

In 2 months, at the end of the exposure, the biochemical and cytologic indicators of injury measured in lavage fluid had all returned to control levels and histologic changes were resolving. There was an increase in the mean linear intercept of alveolar walls and this remained elevated at 6 and 12 months after the exposure (Figure 3). By one year after initiation of the exposure, a qualitative increase in alveolar size was observed but no septal damage or thickening was present as observed by light microscopy (Figure 4).

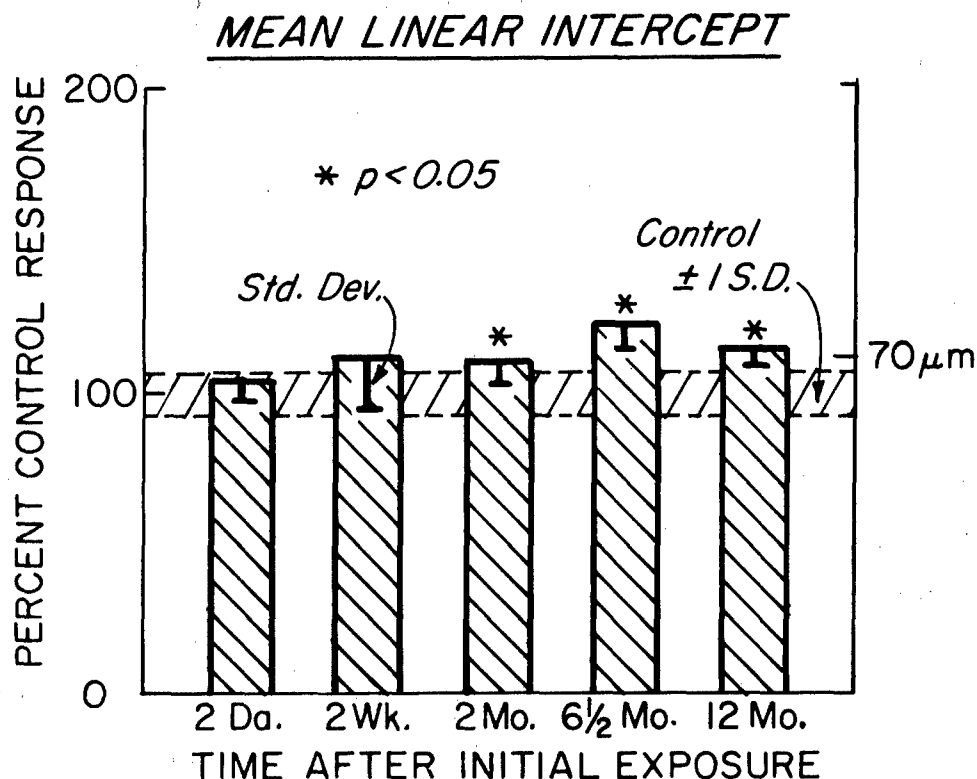


Figure 3. Mean linear intercept of alveolar walls of Fischer 344 rats exposed to 20 ppm  $\text{NO}_2$  for eight weeks. Mean linear intercept is shown as an absolute value and a percent mean control response for rats exposed to 20 ppm  $\text{NO}_2$  and control rats (cont.  $\pm$  S.D.) and sacrificed 2 days-12 months after initiation of exposure. Error bars indicate 1 standard deviation. Increases from mean control response are noted for  $p < 0.05$  using The Mann-Whitney U statistic.

An attempt was made to measure the proteolytic activity of lavage fluid but the assay used (Barrett, 1980) was not sensitive enough for the diluted activity in the lavage fluid. However, tissue levels of acid and neutral proteolytic activity were both increased at 2 days and at 2 weeks and the neutral protease level remained elevated at 12 months after initiation of the exposure (Figure 5).

### DISCUSSION

In lung washings from the acutely injured animals, there was a good correlation between the biochemical and cytologic indicators of pulmonary injury and the degree of injury seen morphologically. The best early indicator of the acute multifocal pulmonary injury caused by  $\text{NO}_2$  exposure was the increased number of neutrophils in both the Syrian hamster and the Fischer 344 rat. The lowest level of this type of injury which could be detected by lung washings was not well determined by this study, since the lowest level of exposure (48 hours at

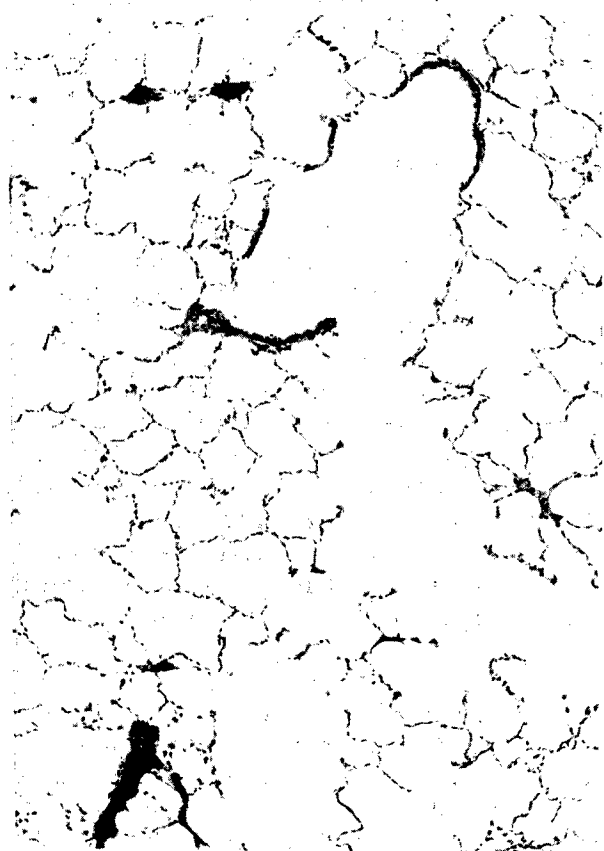


Figure 4. Normal alveolar septa in lungs of rats exposed to  $\text{NO}_2$  and sacrificed at 12 months after initiating exposure (63 x).

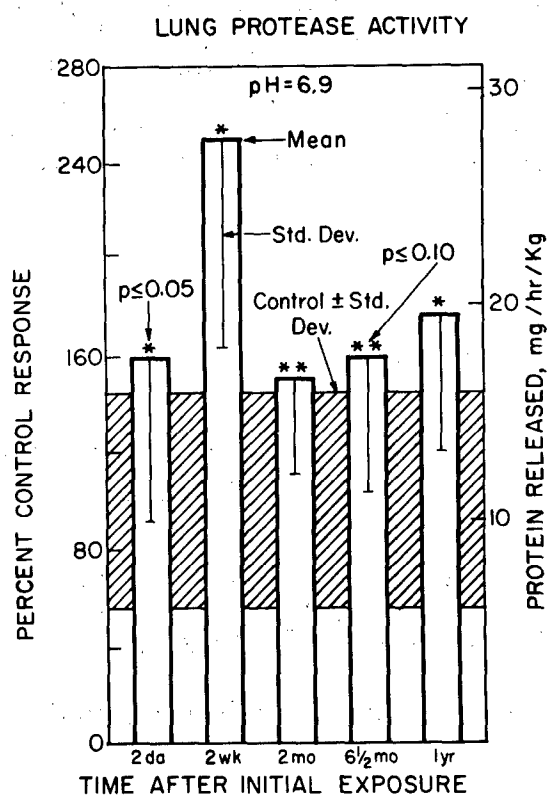


Figure 5. Tissue neutral protease levels in the lung tissue of Fischer 344 rats exposed for 8 weeks to 20 ppm  $\text{NO}_2$ .

12 ppm NO<sub>2</sub>) produced a 10 fold increase of neutrophils in the hamster lung washings. It would appear that much lower levels of injury might have been detected. These results are in contrast with observations in lung washings from Syrian hamsters exposed to CdCl<sub>2</sub> (Henderson et al., 1979). In the diffuse lung injury caused by the metal salt, the biochemical and cytologic parameters were equally sensitive indicators of the degree of injury.

In the study designed to follow changes in lavage fluid parameters which might be indicative of developing chronic pathology, an adaptive response was seen. The adaptation seen histologically was paralleled by indications of adaptation seen in the lung washings. By 2 months, the biochemical and cytologic indicators of the early inflammatory response to injury had all returned to normal and histologic indications of inflammation were also gone. The one biochemical parameter which had not returned to normal by 12 months was the elevated tissue level of neutral proteolytic activity. This correlated well with the histologically observed increase in the mean linear intercept of alveolar walls which was present from 2 to 12 months after initiation of the exposure. Our current efforts are aimed at developing more sensitive and specific assays of proteolytic activity so we can measure the low levels found in the lung washings. This ability would enhance the usefulness of analysis of lung washings as a screening tool for pulmonary injury.

In summary, analysis of lung washings can be used to screen for early inflammatory responses in the lung to acute inhalation exposure to test materials and to measure adaptation to continued exposures. This rapid screening technique can be used to rank the pulmonary toxicity of a series of compounds. As used in our studies, the lung washings are performed on excised lungs as a more rapid method of detecting levels of injury than the use of complete histopathologic examination. The screen is useful for choosing appropriate compounds and exposure levels for subsequent in-depth studies in which complete histopathologic evaluations will be made. In larger animals, the technique could be modified to use segmental lung washings performed serially in the same animal. Our results to date indicate that the lung washings may also prove to be useful in the detection of developing chronic lung pathology if specific and sensitive assays for critical parameters are developed.

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MORPHOLOGY AND FUNCTION OF ALVEOLAR MACROPHAGES  
AFTER EXPOSURE TO OXIDANT GASES

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INTRODUCTION

Bronchiolitis can be experimentally induced in a variety of animals by the inhalation of ambient levels of ozone (Castleman et al., 1980; Mellick et al., 1977; Schwartz et al., 1976). A major component of this bronchiolitis is the accumulation of inflammatory cells consisting of macrophages, eosinophils, and neutrophils. If the ozone insult persists for a period of weeks to months, the inflammatory cell infiltrate also persists, even though evidence of pulmonary epithelial cell necrosis and repair is gone (Castleman et al., 1980; Eustis et al., 1978).

We have attempted to measure the magnitude of this inflammatory cell infiltrate to establish the dose response characteristic for ozone. A quantitative approach was necessary to allow comparison of various ozone exposure regimens and to allow comparison of the response induced by ozone with that of various other inhaled pollutants. Two relatively simple morphologic approaches will be described.

In addition to quantifying the numbers of macrophages accumulating within the lesion site, it is crucial to assess the functional characteristics of these cells. It is not our intent to review all functions of the alveolar macrophage and known effects of various pollutants, but rather to focus on four selected in vitro functions that we have used to gauge the type and magnitude of ozone damage.

Features commonly used to identify macrophages are listed in Table 1. A single feature cannot be used alone to definitely identify a macrophage, but the presence of several features collectively adds to the evidence that a cell is truly of the mononuclear phagocytic series. The macrophage also manufactures and releases substances that have enzymatic capabilities, function as components of host defense systems, and/or alter functions of other cell populations (vanFurth, 1975). Features or functions that we have assessed either during or after ozone insult include proliferation and colony-forming capacity, migration ability, phagocytic ability, and plasma membrane receptors.

TABLE 1. FEATURES USED IN IDENTIFICATION OF  
ALVEOLAR MACROPHAGES

1. Glass adherence
2. Abundant cytoplasm
3. Numerous surface membrane ruffles and pseudopodia
4. Numerous lysosomes which vary in density and size
5. Presence of C<sub>3</sub>b and Fc plasma membrane surface receptors
6. Phagocytic and pinocytotic activity

MORPHOLOGIC EVIDENCE OF OZONE-INDUCED  
ALVEOLAR MACROPHAGE ALTERATION

Studies of lungs after ozone insult have clearly demonstrated an increase in numbers of macrophages within proximal alveoli of the centriacinar region of the rat (Boorman et al., 1980), or within alveolar outpockets of respiratory bronchioles of the nonhuman primate lung (Eustis et al., 1978; Mellick et al., 1977). Evaluation of tissues by light microscopy or scanning electron microscopy, using nonquantitative approaches, demonstrated the presence of macrophage infiltrates even after exposure to only 0.2 ppm ozone for seven days. Intraluminal macrophages contained frequent vacuolated phagolysosomes, membrane ruffles, and filopodia. Occasional mitotic figures could be observed during early phases (36-50 hours) of the ozone insult (Castleman et al., 1980).

Our approach to quantitation of the inflammatory infiltrate has been of two types. Because of the focal nature of the ozone-induced lesion, we have not used the conventional stereological approach described by Weibel (Weibel et al., 1966; Weibel and Knight, 1964). The procedure that we have applied to rat lungs is completed using scanning electron microscopy (SEM), either by directly viewing the screen or from photomicrographs (Brummer et al., 1977), in order to count the number of inflammatory cells per alveolus in centriacinar regions. This procedure is best applied to species not having long, branching respiratory bronchioles. Alveoli, defined as saccular outpockets of alveolar ducts lined by only Type 1 or Type 2 alveolar epithelium, were counted distal to the termination of the bronchiolar epithelium. Intact cellular structures within these alveoli were counted as inflammatory cells. Figure 1 summarizes the data collected from this type of evaluation procedure. This quantitative approach indicated that the magnitude of inflammatory cell infiltration was greater in rats exposed to 0.8 ppm ozone than those exposed to 0.2 ppm. The lesion was most severe at seven days of exposure; it regressed in severity with increased length of exposure but persisted at a level approximately 4-fold greater than the control. Using this procedure, quantitative differences were not demonstrated between the 0.5 ppm ozone-exposed group and the other two exposure levels.

In species with long branching respiratory bronchioles, the SEM approach is more difficult; since to identify sites accurately for sampling a detailed dissection of the airways is required. An alternative approach is to use H and E stained 7-micron thick paraffin sections (McQuillen et al., 1977). The surface length of the exposed airway surface starting at the terminal bronchiole can be measured using various methods of image analysis (Zeiss, MOP-3). Within this known length of airway the number of inflammatory cells can be counted, as well as differentiated



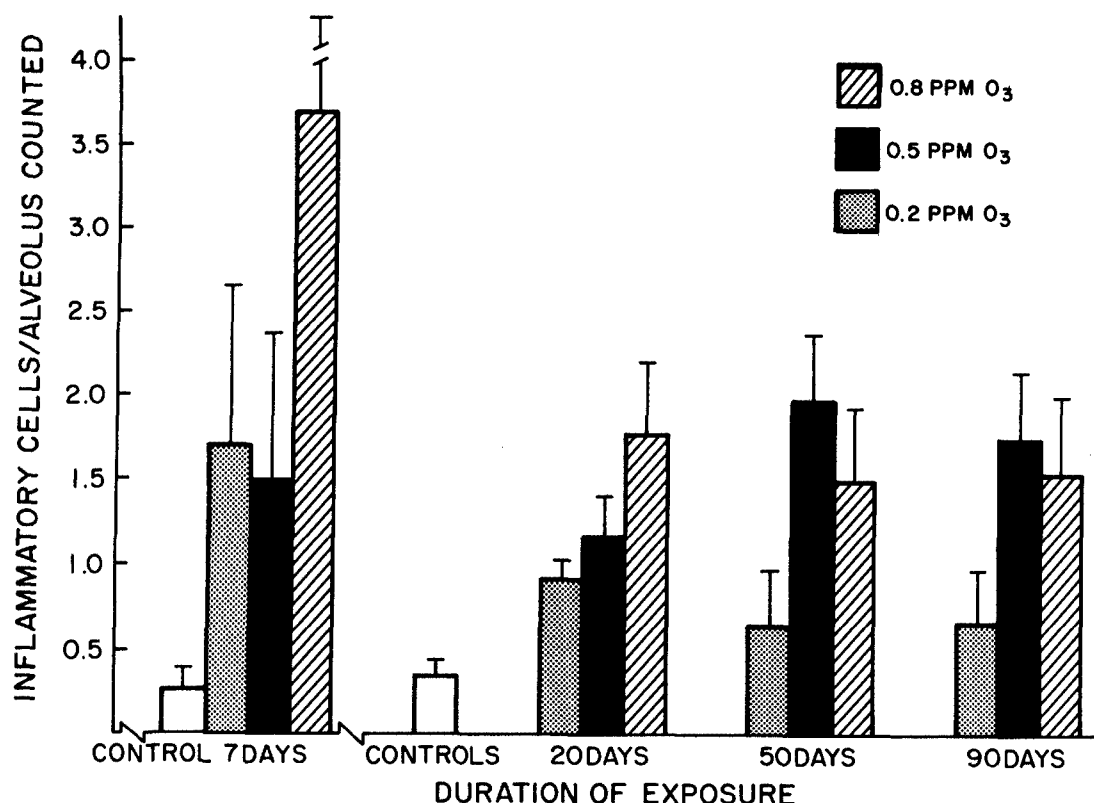


Figure 1. Number of intraluminal inflammatory cells per alveolus were counted in centriacinar regions of the rat lung after ozone exposure. A minimum of five centriacinar regions was counted from each rat, and there were three rats per exposure group. Six rats served as controls at day 7, and 18 rats served as controls for the 90-day exposure.

as to cell type. This approach allows comparison between exposure groups on a basis of inflammatory cells per millimeter of bronchiolar surface length (Figure 2).

This procedure completed on sections of monkey lungs also demonstrated that the inflammatory cell infiltrate was most severe on day 7 of exposure and that it regressed with time. The infiltrate on day 7, after exposure to 0.8 ppm ozone, was approximately 20-fold greater than the control value. It was 11-fold greater in monkeys exposed to 0.5 ppm ozone for 7 days. Values at 90 days of exposure for 0.8 or 0.5 ppm ozone were 10-fold and 9-fold, respectively, above controls.

As an index of morphologic change induced in macrophages by ozone insult, we also attempted to measure cell spreading on glass coverslips. Free lung cells collected by lavage were allowed to adhere in culture to glass coverslips for 3 hours. Nonadherent cells were removed by vigorous washing and discarded. Cells from rats exposed to 0.8 ppm ozone for 3, 7, or 20 days were prepared in this fashion, fixed, and prepared for SEM examination. Cells were randomly selected during SEM examination and photographed at a standard magnification, specimen tilt, and KV. The area enclosed by the perimeter of each cell was measured, using photomicrographs and the Zeiss MOP-3 image analyzer. This

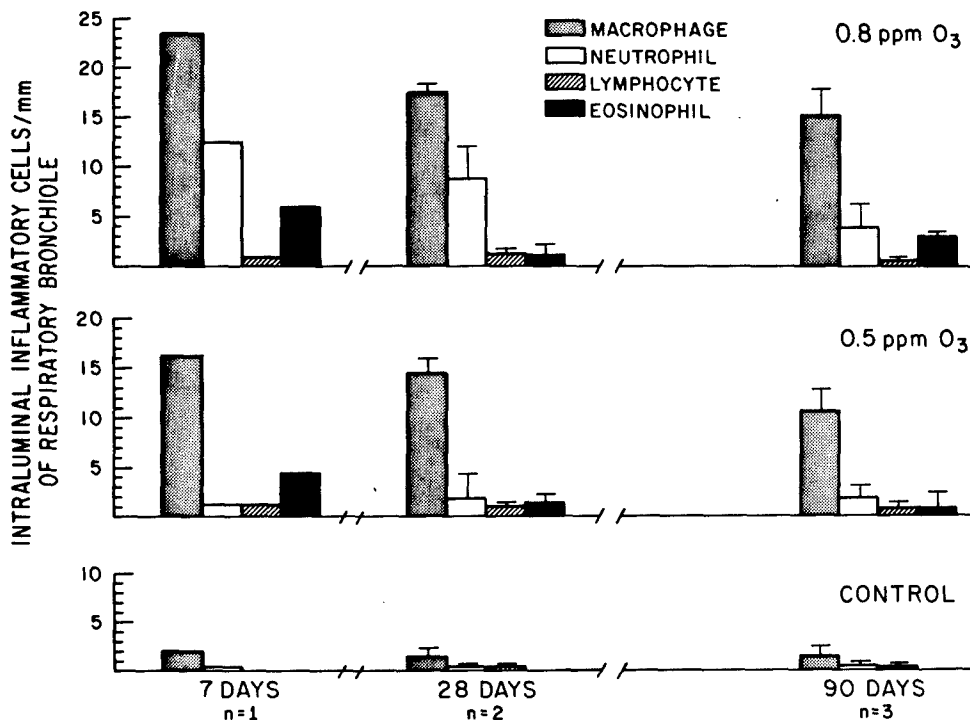


Figure 2. The number of inflammatory cells per mm of surface distance was determined in a group of bonnet monkeys exposed to 0.8 or 0.5 ppm ozone for 7, 28, or 90 days. Using this procedure, a differential cell count can also be completed to characterize the type of inflammatory cell infiltrate.

approach demonstrated a significant ( $P \leq 0.001$ ) increase in spreading of macrophages obtained from rats exposed to ozone for 3 days but not in macrophages obtained from rats exposed for 7 or 20 days (Figure 3). The length of time in culture after the initial adherence period influenced this observation, since macrophages left in culture for 24 hours (plus 3 hours for adherence) after the termination of exposure did not demonstrate a difference between control and exposed cells in the degree of spreading.

#### FUNCTIONAL ALVEOLAR MACROPHAGE ALTERATIONS INDUCED BY OZONE INSULT

The lung is one of few organs that lends itself to repeated direct sampling with little harm to the host. Free lung cells can be easily collected by bronchoalveolar lavage (Schwartz and Christman, 1979). Several procedures have been described using either double-lumened ballooned catheters or fiberoptic bronchoscopes. The lavage procedure has the advantage of being nonterminal and repeatable during multiple phases of an experiment. Caution must be used in selecting an anesthetic agent (Fisher et al., 1978), and the time interval between lavage procedures should generally be no less than 14 to 20 days. Lavage is also commonly used in terminal experiments.

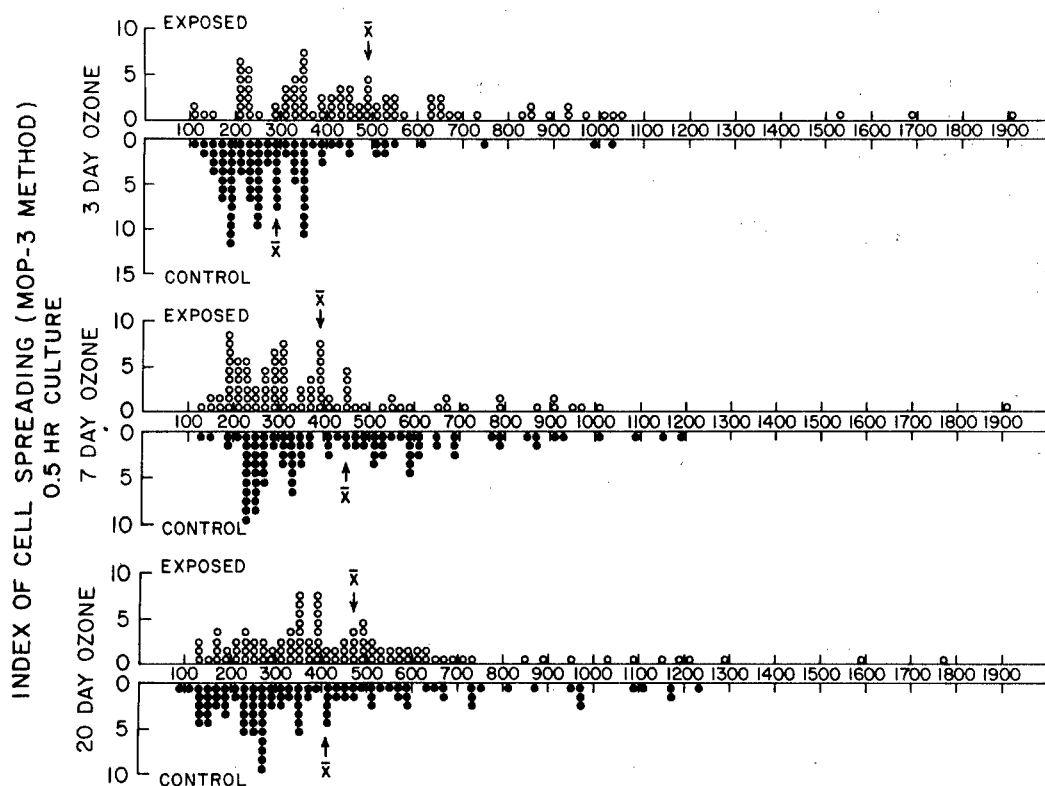


Figure 3. Scattergram of alveolar macrophage spreading index. Each circle represents a relative in vitro measurement of the glass surface area covered by the spreading cytoplasmic processes of a single macrophage. The population mean is indicated by the  $\bar{x}$ . Macrophages were collected by bronchoalveolar lavage after exposure of rats for 3, 7, or 20 days to 0.8 ppm ozone. The spreading index of exposed cells is significantly increased above control cells after 3 days of exposure but not after 7 or 20 days.

Both the acellular and cellular components of the lavage material can be of value in assessment of pulmonary damage. Our focus has been on the cellular component. Initial characterization must include determination of numbers of cells collected, the types, and their viability. Selected purification procedures may be desirable. Our observations relative to the effects of ozone have focused on colony formation and proliferation potential of free lung cells, mobility, phagocytosis, and plasma membrane receptors.

#### COLONY FORMATION AND MACROPHAGE PROLIFERATION

The alveolar macrophage retains a potential for local proliferation, and this capability may contribute to the total accumulation of macrophages during pulmonary insult (Boorman et al., 1979; Boorman et al., 1979). The formation of colonies in a semisolid medium has been used as an index of progenitor cells

which can serve as a local proliferation source. The precursor cells of these colonies are thought to be common hematopoietic progenitors for monocyte-macrophages and granulocytes. The procedure for demonstrating colony forming units in culture (CFU-C's) is outlined in Figure 4. Boorman and co-workers (1979) demonstrated that CFU-C's significantly increased above control values during the first 48 hours of ozone exposure in rats, but declined to near normal values after 7 days of exposure (Figure 5). This observation is important since it indicates that during periods of greatest demand and pulmonary injury, additional inflammatory cells may originate locally from progenitor cells. The prolonged culture period (22 days) required to form cluster or colonies of cells in semisolid media provides a serious disadvantage, and other methods are available that allow quicker determination of cell proliferation capabilities. The advantage of colony formation is the ability to differentiate cell types forming within the colony, as both monocyte-macrophage or granulocyte colonies can be identified. This differentiation has not been completed with ozone-exposed free lung cells.

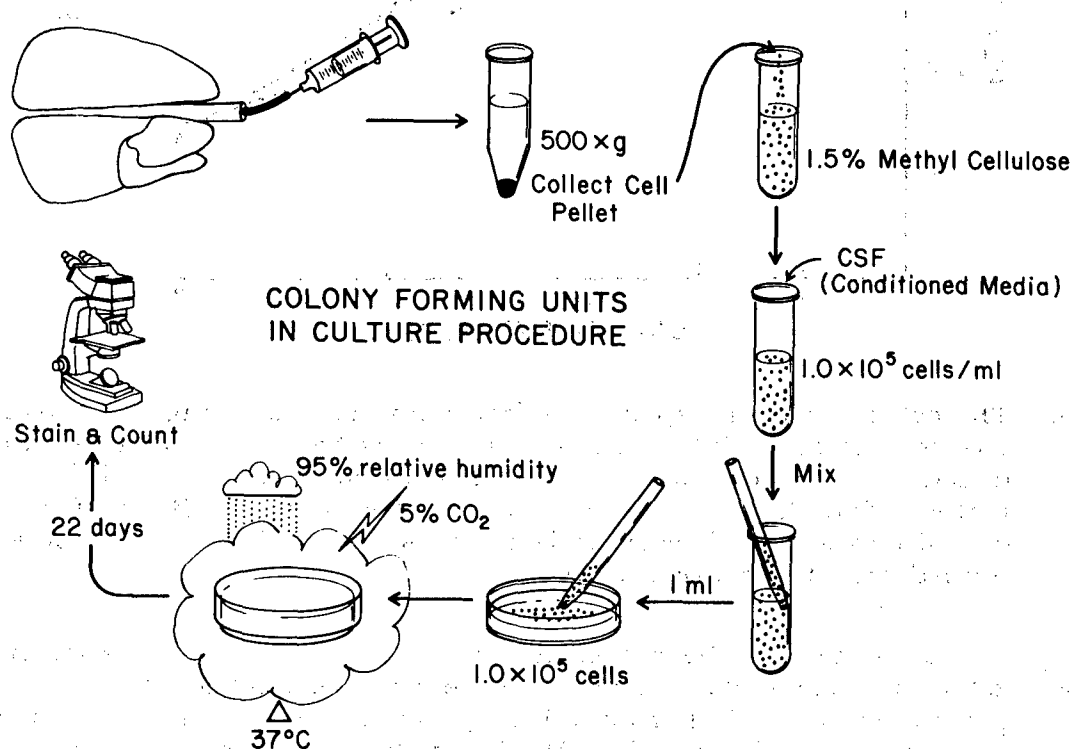


Figure 4. The colony forming units in culture procedure involves collection of cells from the lung, distribution of a known number of cells in methyl cellulose and the addition of an appropriate colony stimulating factor(s) (CSF). This procedure is described in detail by Boorman and co-workers (Boorman et al., 1979; Boorman et al., 1979).

### Colony Forming Capacity of Free Alveolar Cells Lavaged from O<sub>3</sub> Exposed Rats

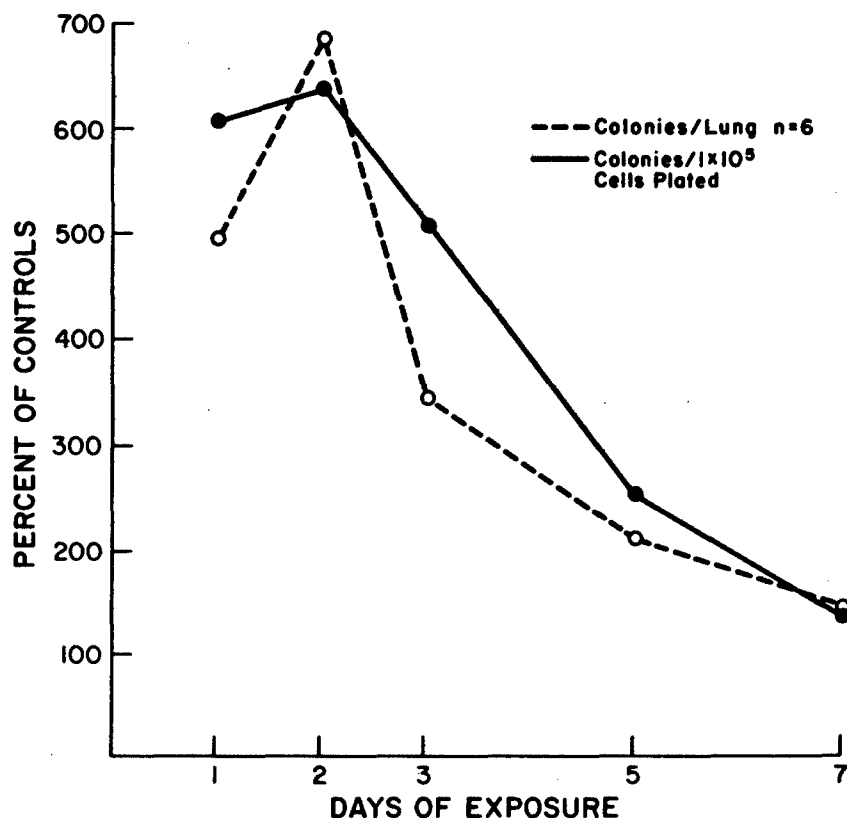


Figure 5. The colony forming capacity of free lung cells following ozone exposure (percent of controls). The free lung cells from 41 control rats produced an average of  $20 \pm 5$  (mean  $\pm$  SEM) colonies per  $1 \times 10^5$  cells plated. Free lung cells from 14 exposed rats were cultured for each day of exposure. A mean of 72 colonies per total lavage sample were formed from the control lungs.

Cell proliferation has also been measured using <sup>3</sup>H-thymidine incorporation into newly formed nucleic acid (Figure 6). Free lung cells collected from rhesus monkeys began to increase <sup>3</sup>H-thymidine incorporation above a 24-hour baseline value by 3 days in culture (Figure 7). Maximal values occurred at 5 days in culture. Conditioned media, obtained from other rhesus monkey alveolar macrophage cultures as a potential source of colony stimulating factor, did not appear to enhance <sup>3</sup>H-thymidine incorporation above values obtained from cultures that did not receive this conditioned media. Preliminary results using free lung cells collected from rhesus monkeys continuously exposed to 0.8 ppm ozone for 1, 2, or 3 days are illustrated in Figure 8. Control values are mean values obtained from eleven unexposed rhesus monkeys.

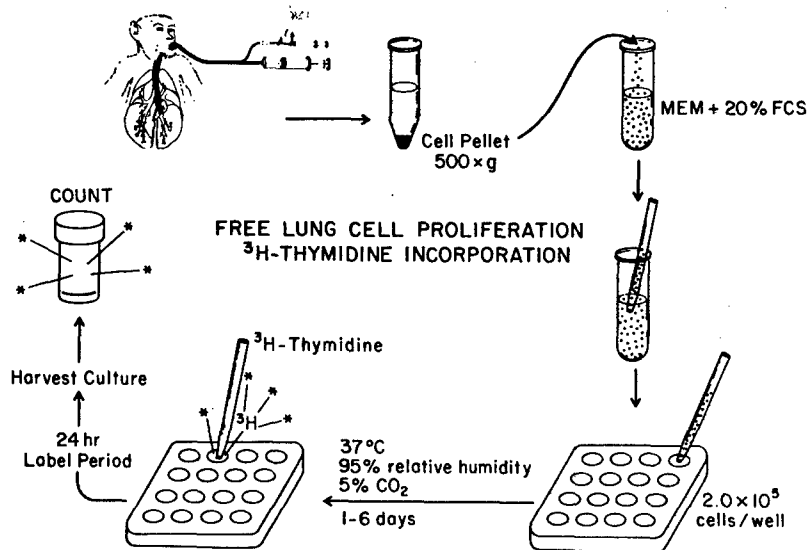


Figure 6. Incorporation of  $^3\text{H}$ -thymidine by free lung cells after collection by bronchoalveolar lavage provides another means to assess the proliferative potential during periods of pulmonary injury.

Influence of Conditioned Media (Monkey AM 10 Day Culture Supernatant) on Free Lung Cell  $^3\text{H}$ -Thymidine Incorporation

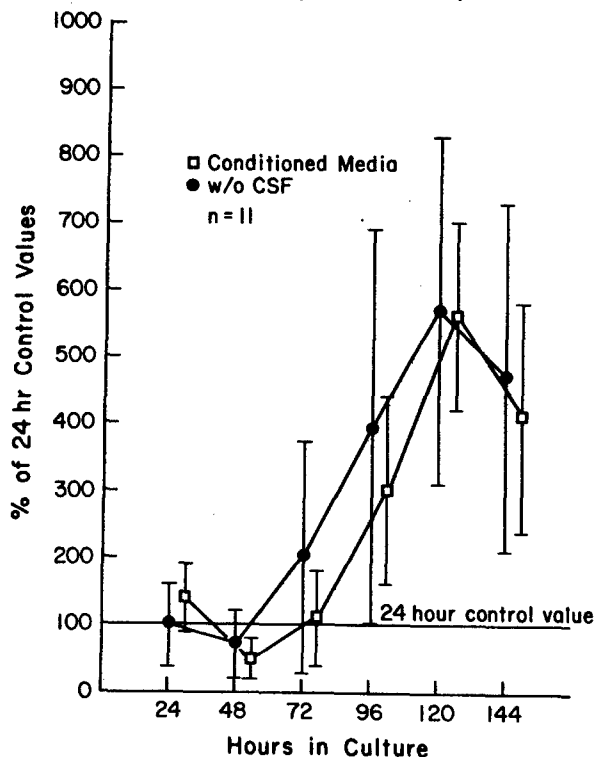


Figure 7. Free lung cells were collected by bronchoalveolar lavage from eleven rhesus monkeys. Incorporation of  $^3\text{H}$ -thymidine remained relatively constant until 72 to 96 hours in culture. Macrophage conditioned media (CSF) did not significantly stimulate  $^3\text{H}$ -thymidine incorporation, but did remove some of the variability in the response.

### Influence of O<sub>3</sub> Exposure on Free Lung Cell <sup>3</sup>H-thymidine Incorporation

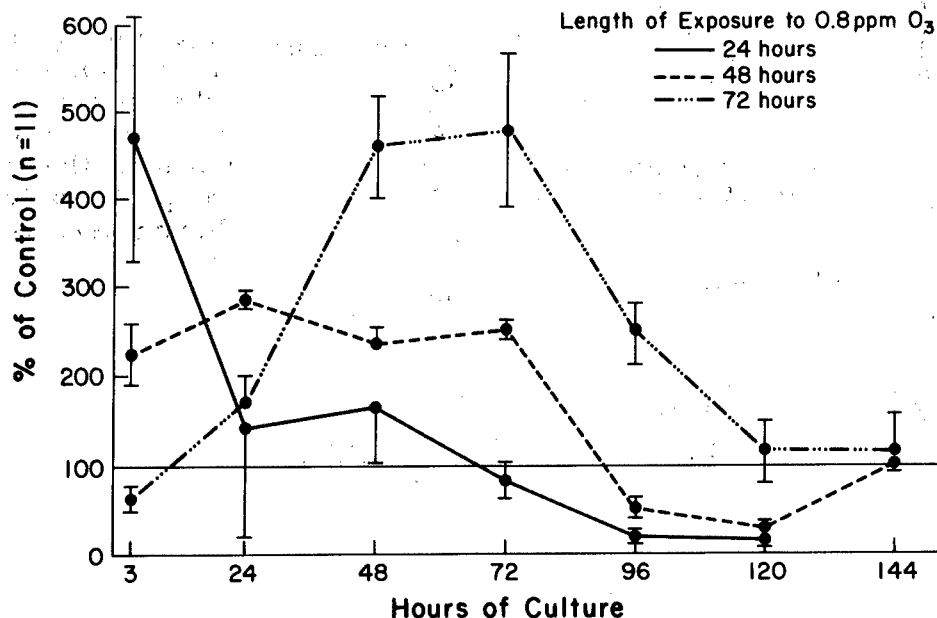


Figure 8. Preliminary results obtained from the exposure of rhesus monkeys to 24, 48, or 72 hours of 0.8 ppm ozone. Control values were obtained from cells collected from nonexposed rhesus monkeys and placed in culture for 3, 24, 48, 72, 96, 120, or 144 hours. Each time period served as its own control. The difference in response at each exposure length most likely reflects the actively changing cell population with different proliferative potentials during this early phase of lung damage.

Results indicated that the proliferative rate of cells collected after 24 hours of in vivo ozone exposure was markedly elevated above control values but that this rate rapidly returned to near control values and became suppressed with increasing length in culture. Cells obtained from monkeys after 72 hours of ozone insult initially had a slightly suppressed proliferative response but did respond in a delayed fashion after 48 and 72 hours in culture. These results are preliminary and must be confirmed; however, they do suggest an effect on macrophage population during pulmonary insult and evolution of the resulting lesion.

#### MOBILITY

Mechanisms responsible for recruitment and accumulation of macrophages within the lung must include local proliferation, chemotaxis and migration inhibition. The latter two features center on the mobility of these inflammatory cells. Several in vitro procedures are available to measure cell locomotion (Gallin and Quie, 1978). The system that we have selected is the underagarose migration assay described by Nelson and associates (Gallin and Quie, 1978). We have used this system to assess changes in the influence of ozone insult on the random

migration of alveolar macrophages (Schwartz and Christman, 1979). Alveolar macrophages obtained from lungs of rhesus monkeys after they breathed ozone for 7 days demonstrated decreases both in the number of cells randomly migrating from wells cut in agarose plates and in the maximal distance of the leading front of migrating cells. This observation is important in that it implies that ozone exposure impairs alveolar macrophage migration that may be a direct cytotoxic effect of ozone or secondary to the release of migration inhibitory mediators.

## PHAGOCYTOSIS

Phagocytosis of infectious or noninfectious particulates is a critically important function of the alveolar macrophage that directly relates to pulmonary defense. A decreased ability of rabbit alveolar macrophages to phagocytize group C *Streptococcus* sp. in vivo was observed by Coffin and co-workers (1968) after exposure of rabbits to 0.6 to 9.5 ppm ozone for 3 hours. After exposure of rabbits to 3.0 to 5.0 ppm ozone for 3 hours Huber and associates (1971) reported morphologic changes which included fenestration of the plasma membrane, dilatation of endoplasmic reticulum, and formation of autophagic vacuoles indicating severe cell damage and death. Exposure to lower levels of ozone (0.2 to 0.8 ppm) did not result in these severe morphologic changes, but of more importance is the concept that as exposure length is increased the cell population changes, and the pulmonary defense capabilities may also differ from those of both the control population and the cell population that was present during the early phases of insult.

In a study to assess the phagocytic capability of the alveolar population during longer-term exposures a group of rats were exposed to 0.8 ppm ozone for 3, 7, or 20 days. Alveolar macrophages were collected by bronchoalveolar lavage, allowed to adhere to glass coverslips, and incubated with carbon-coated latex microspheres. The percentages of phagocytic cells were determined by light microscopy after 0.25, 0.5, 1, 2, 4, 8, or 24 hours of incubation (Christman and Schwartz, 1981). Results are summarized in Figure 9. An enhanced phagocytic activity was observed after ozone exposure, with the greatest increase being on day 3 of exposure. This enhanced phagocytic activity correlated with an increase in cell spreading (Figure 3). This observation was in contrast to the impaired phagocytic function after only a few hours of exposure.

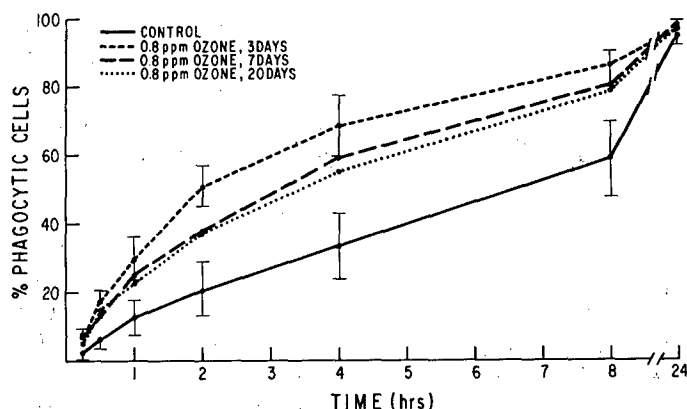


Figure 9. The time course of phagocytosis by alveolar macrophages demonstrated that alveolar macrophages obtained from ozone exposed rats phagocytized carbon-coated latex microspheres more rapidly than control macrophages. The bar-enclosed lines represent standard deviations.



## PLASMA MEMBRANE RECEPTORS

Cell surface receptors such as Fc and  $C_3b$  enable the alveolar macrophage to recognize certain particles selectively including bacteria, that have been opsonized with immunoglobulins and/or complement. These receptors facilitate identification and attachment of particulates by the alveolar macrophage. Fc and  $C_3b$  receptors are not located randomly on the plasma membrane, but are principally observed on thin cellular membrane extensions. The process of particle attachment and phagocytosis proceeds differently depending on the surface receptor involved (Kaplan, 1977). Attachment to the Fc receptor involves formation of a cuplike cytoplasmic extension around the particle, followed by endocytosis. Particles bound to the  $C_3b$  receptor appear to be directly endocytosed in the absence of plasma membrane extensions. The  $C_3b$  receptor mediates only attachment with normal macrophages, but with activated macrophages this receptor also mediates phagocytosis (Bianco et al., 1975).

We have assessed changes in alveolar macrophage Fc,  $C_3b$ , and IgM receptors during a prolonged ozone insult of bonnet monkeys (*Macaca radiata*). Monkeys were exposed to 0.8 ppm ozone (8 hours/day) for 3, 7, 60, 90, 120, 180, 270, or 360 days. Alveolar macrophages obtained by bronchoalveolar lavage were cultured on glass coverslips in serum-free medium. Nonadherent cells were washed off after one hour of culture, and suspensions of sheep erythrocytes were added to the cultures, either uncoated or coated with rabbit anti-sheep erythrocyte IgM or IgG or rabbit anti-sheep erythrocyte IgM plus  $C_5$ -deficient AKR mouse serum. Attachment and/or phagocytosis of these erythrocyte complexes by alveolar macrophages were determined by light microscopy. Results are summarized in Figure 10. We observed that bonnet monkey alveolar macrophages did not spontaneously rosette with sheep erythrocytes. No IgM receptor was observed. Fc and  $C_3b$  receptors were observed, and  $C_3b$  mediated attachment and phagocytosis was significantly increased at 3 and 7 days of exposure (Figure 10A). A difference was not observed in alveolar macrophage Fc receptor attachment or phagocytosis except for an increase at 120 days (Figure 10B). These changes may serve as sensitive indicators of membrane damage or perturbation during the early phase of ozone insult.

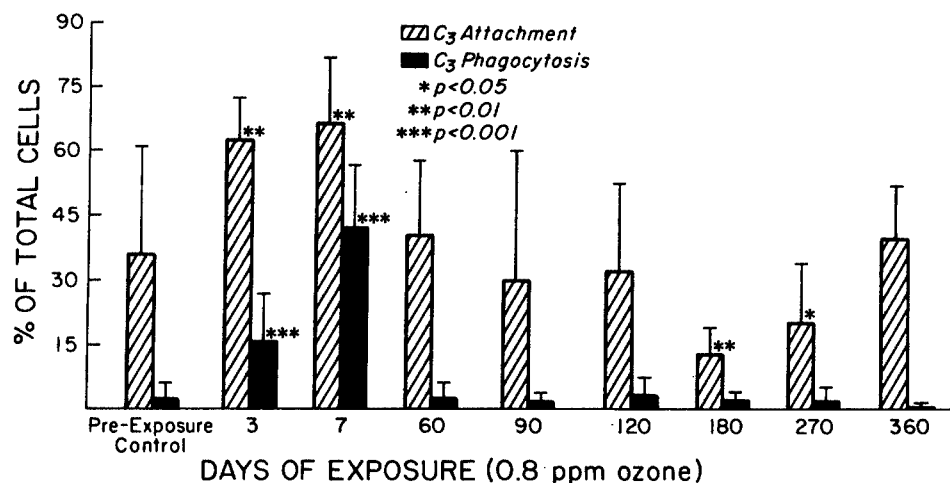


Figure 10A. Ozone-induced alterations in plasma membrane receptors of alveolar lavage from bonnet monkeys during exposure to 0.8 ppm ozone.  $C_3b$  membrane receptors increased significantly during the early phase of exposure, as did  $C_3b$  mediated phagocytosis.

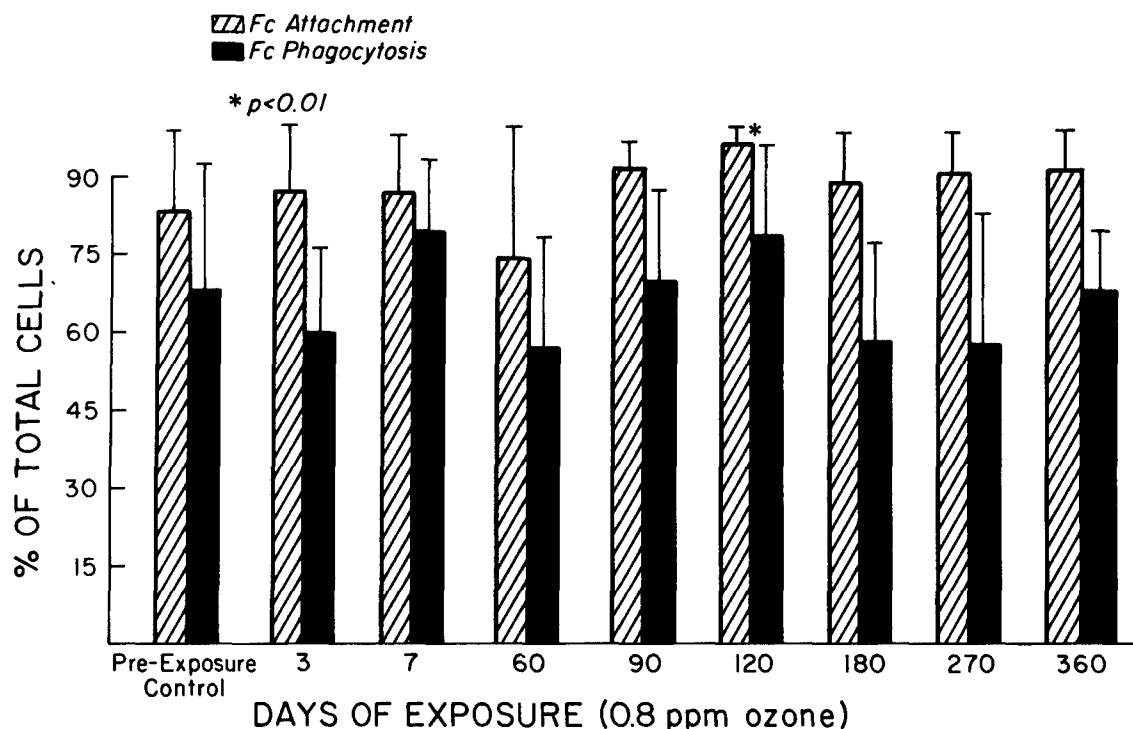


Figure 10B. Ozone-induced alterations in plasma membrane receptors of alveolar macrophages were observed. Cells were collected by bronchoalveolar lavage from bonnet monkeys during exposure to 0.8 ppm ozone. Changes in Fc plasma membrane receptors were not as obvious, suggesting selected alteration of cell surface receptors by ozone.

### SUMMARY

It is clear that ozone insult produces both morphologic and functional changes in the alveolar macrophage population. Quantitative approaches are crucial if proper assessment of the magnitude, persistence, and irreversibility of pulmonary disease induced by ambient levels of ozone insult are to be determined. Studies completed in nonhuman primates have demonstrated a 4- to 5-fold increase in the number of alveolar macrophages which persist within small airways for periods up to three months during continual insult. Events leading to this increased population of alveolar macrophages are poorly understood, but evidence indicates that mobility and proliferative potential are significantly altered. Plasma membrane receptors, phagocytic ability, and cell size all appear to be influenced. Additional studies will continue to unfold the mechanisms of ozone-induced pulmonary damage, and the significance of this type of damage on the pulmonary defense systems and lung structure.

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COLLAGEN AND MUCOPOLYSACCHARIDE SYNTHESSES AFTER  
ACUTE AND CHRONIC EXPOSURE TO OXIDANT GASES

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I bring what is probably an unusual lack of background and training to the area of lung toxicology, being a born-again molecular biologist formally trained in chemistry and biochemistry. That sort of through the back door entry into a field encourages one to hold heterodox views. I'd like to present some data today that suggest to me that some of our most cherished dogma, like the Emperor's New Clothes, may exist only in the eye of the beholder. I'll come back to this point at the end of my talk, and be a bit more specific then.

We have used the tracheal explant system, developed several years ago in the laboratories of Dr. Lynn Reid (1970) and others (Boat et al., 1971; Bonnanni et al., 1973; Yeager et al., 1971; Chakrin et al., 1972; Ellis and Stahl, 1973; Formijne et al., 1964), as a quantitative assay for monitoring effects of air pollutants and other pneumotoxins on the respiratory epithelium. We use an "in vivo" approach, featuring exposure of animals (usually rats) in vivo to known amounts of pneumotoxin, followed by removal of their tracheae for assay of effects in vitro. With small animals such as rats, we routinely incubate the tracheae in tissue culture medium containing radioactively labeled precursors. The culture medium is then removed and an aliquot is treated with 5% trichloroacetic acid to precipitate the proteins present. With a suitable choice of labeled precursor and incubation time for the rat tracheal explants, essentially all of the acid-precipitable radioactivity in the medium is mucus glycoprotein (Jennings et al., 1977; Last et al., 1977). What are the characteristics of such an assay? The appearance of acid-precipitable glycoproteins in the culture medium can first be observed after about three hours. The incorporation rate is linear between about 8 hours of incubation and at least 96 hours. For routine assay purposes we generally incubate tracheal explants for about 24 hours. This is a convenient duration of incubation in that enough radioactivity is incorporated to enable accurate quantitation, and the incorporation is clearly linear with respect to time, so that an actual rate of incorporation is being measured.

How can we apply this system to air pollution toxicology? Figure 1 illustrates the results of an experiment in which rats were exposed to 0.8 parts per million of ozone for eight hours per day for periods ranging from 1 to 90 days of exposure, and their response was elevated by the tracheal explant assay. There is an initial decrease in the rate of glycoprotein secretion by tracheal slices as compared to the rate of secretion by slices prepared from matched control rats.

This decrease lasts for two to three days, as evaluated by statistical significance; the secretion rate once again returns to the control values after a period of about five to ten days (in different experiments). If the rats are exposed to ozone for periods longer than about a week, the secretion rate seems to increase continuously with longer durations of exposure, up to 90 days in Figure 1, and up to 180 days of exposure in other experiments we have performed. The hatched bar at 30 days shows the results of a separate exposure performed some 6 months after the one that had given rise to the rest of the data presented in Figure 1.

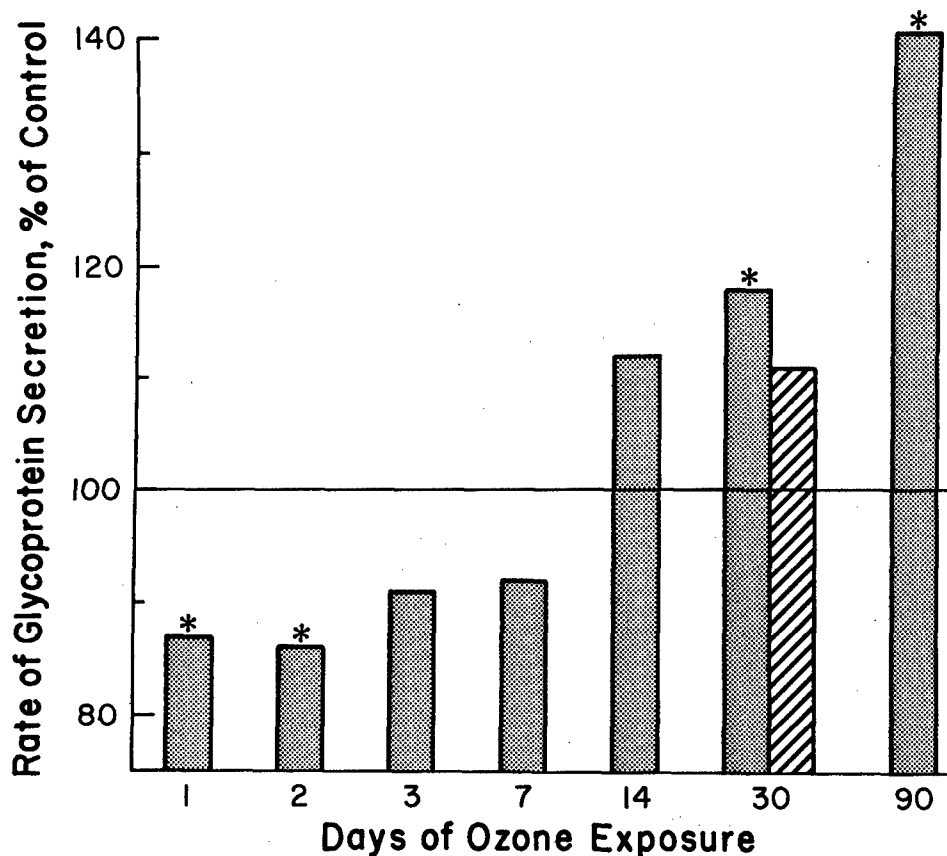


Figure 1. Glycoprotein secretion rates (expressed as percent of control value = 100%) of tracheal explants prepared from intact rats exposed for eight hours per day to 0.8 ppm of ozone for the lengths of time indicated on the abscissa. The hatched bar at 30 days represents results from another experiment to indicate the reproducibility of the results obtained by this technique.

The tracheal explant system is sensitive not only to the duration of exposure of rats to ozone, but also the concentration of ozone to which the rats are exposed (Last et al., 1977). A statistically significant decrease in secretion rate is observed after exposure for three days to 0.8 parts per million of ozone or to 0.6 parts per million of ozone for eight hours per day. An insignificant decrease is observed at 0.4 parts per million and no effect is seen at 0.2 parts per million. Comparisons between matched controls and experimental rats are very precise within a given experiment. In a single experiment the size of tissue slices and the matching of rats can be controlled rigorously. A single batch of medium can be used so there are minimal errors introduced by pipetting and addition of labeled precursor to the medium. On the other hand, comparisons between separate experiments are much more difficult. Small differences in technique from day to day result in variations in the absolute value for control rats such that data within a chronic exposure regimen must usually be normalized to control values; hence, the expression of the data in Figure 1 as percent of control. In addition, growth of the rats within a given chronic exposure experiment is appreciable and can also affect these results, as can also expression of viral disease and a host of other parameters we do not fully understand.

The tracheal explant system can be used with animals other than rats. We have demonstrated elsewhere (Jennings et al., 1977) that tracheal explants from a wide variety of experimental animals, as well as human tracheal and/or bronchial explants, can be used in this system. For example, we have used this system for studying effects of exposure of monkeys to ozone, finding results that complement our studies with rats. For example, in preliminary experiments using tracheal slices prepared from bonnet monkeys (*Macaca radiata*) exposed to 0, 0.5, or 0.8 ppm of ozone for seven days, we found a dose-dependence increase in mucus glycoprotein secretion rate for the slices from exposed monkeys (Table 1). Since these data are from multiple slices from individual monkeys at each exposure level, we clearly need to study more animals before we can draw any rigorous conclusions. Nonetheless, we think we are seeing effects of relatively low levels of ozone on tracheobronchial mucus glycoprotein secretion by nonhuman primates, an effect that may be relevant to human exposures.

TABLE 1. GLYCOPROTEIN SECRETION RATES (MEAN  $\pm$  1 SD)  
FOR TRACHEAL SLICES FROM MONKEYS  
EXPOSED TO OZONE FOR SEVEN DAYS

Controls	0.5 ppm O <sub>3</sub>	0.8 ppm O <sub>3</sub>
12,400 $\pm$ 2,100	16,700 $\pm$ 3,800	21,900 $\pm$ 6,400

Naturally, demonstration of quantitative changes in airway explant secretion rates raises the question of whether qualitative changes are occurring concomitantly. We have some preliminary evidence with rats that ozone exposure may indeed cause shifts in the types of mucus glycoprotein being synthesized from one category to another. Culture medium glycoproteins secreted by tracheal explants from rats exposed for three days to 0.5 ppm of ozone were examined

by ion-exchange chromatography on DEAE-cellulose. The elution profile for labelled glycoproteins from pooled normal rat tracheae is shown in Figure 2. Peaks A1 and A2 are neutral glycoproteins that apparently differ on the basis of their oligosaccharide side chains (composition and/or length, data not shown). Peaks BI-BIV are acidic glycoproteins, which apparently differ in their relative degree of sulphation of sugar residues. Peak BII seems to contain most of the sialic acid residues in the total acidic glycoprotein fraction, as based upon its binding properties to lectin columns and its susceptibility to digestion with bacterial neuraminidase. When similar columns are analyzed for preparations of mucus glycoproteins from pooled culture media of tracheae from rats exposed to ozone, the results presented in Table 2 are obtained. These data suggest that the decreased tracheal glycoprotein secretion rate observed after exposure of rats to 0.5 ppm of ozone for three days is caused by decreases in a specific glycoprotein fraction BII, the sialylated glycoprotein(s). The relative contents of neutral and of heavily sulphated glycoproteins are increased in the culture medium from exposed rats. I think these data may be giving us a clue as to the cellular basis of upper airway effects elicited by exposure of rats to ozone, but clearly we have a lot more work to do to understand exactly what these types of data mean. It is fascinating that these changes are consistent with concurrent histochemical observations of a shift to more acidic mucosubstances in tracheal epithelial cells from rats in the same study (Moore, 1979).

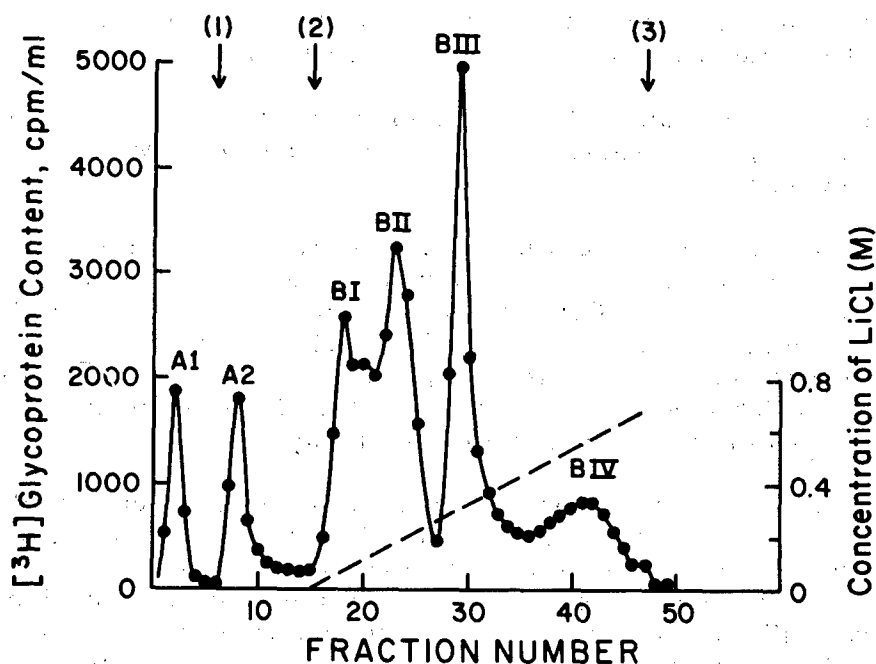


Figure 2. DEAE-cellulose chromatography of papain-solubilized mucus glycoproteins secreted into the culture medium by rat tracheal explants. Arrows indicate: (1) 50 mM acetate buffer wash; (2) application of gradient, and; (3) 2 M LiCl wash. Fraction volume was about 2.2 ml; the radioactivity in a 1.0 ml aliquot was counted (solid line). The LiCl concentration is shown by the broken line, as determined by conductivity measurements.



TABLE 2. COMPARATIVE DEAE-CELLULOSE ANALYSES OF MUCUS GLYCOPROTEINS SECRETED BY TRACHEAL EXPLANTS FROM CONTROL AND OZONE-EXPOSED RATS

<u>Peak</u>	<u>Mean Percentage of Total Peak Radioactivity</u>	
	<u>Control</u>	<u>Exposed</u>
A1 + A2	10.9 ± 0.4	13.8 ± 0.8
B I	12.9 ± 1.2	14.8 ± 1.2
B II	37.9 ± 9.7	24.9 ± 6.5
B III	24.7 ± 7.0	22.0 ± 4.2
B IV	13.6 ± 3.1	24.5 ± 9.3
Ratio $\frac{B}{A}$	8.2 ± 0.3	6.3 ± 0.4
Mucus Glycoprotein Secretion Rate*	61,000	47,000

\* Counts per minute of acid-precipitable radioactivity per ml of culture medium after tracheal slices were incubated for 24 hours.

Data are given as mean ± SD for pooled media from 6-9 slices for each experiment. N, the number of experiments analyzed, was six control and six exposed.

We conclude from these chronic studies that increased mucus glycoprotein secretion rates observed with rat tracheal explants persist for as long as six months of continuous exposure. Comparison of quantitative results at 1 and 3 months provides no evidence for lessening of the effect; that is, there does not seem to be a decreased response to ozone exposure occurring as evaluated by this type of assay, which presumably reflects stable changes during exposure to ozone among cell populations along the large airways.

We have also been studying collagen synthesis by lung parenchymal minces cultured for a few hours in vitro, during which time the amount of collagen synthesized is a linear function of time of incubation. This type of assay presumably tells us what the rate of lung collagen synthesis was in vivo at the moment the rat was killed. We actually have evidence that this is true in another system, in which the in vitro rate of collagen synthesis by lung minces was quantitated and shown to be the same as the in vivo rate, determined by injection of labeled precursor several hours before killing the rats. We have used in vitro methods first described by Crystal and colleagues (1974). When rats were exposed to levels of ozone between 0.5 and 2.0 ppm continuously for one, two or three weeks, the observed collagen synthesis rates by their lungs were increased. We have shown (Last et al., 1979) that the increase observed in the collagen synthesis rate by rat lungs upon ozone exposure is a linear function of the level of ozone

to which the rats are exposed after one, two, or three weeks of continuous exposure. We have also shown that there is an excellent correlation between pulmonary fibrosis as defined by lung collagen synthesis rates and the extent of fibrosis as scored semi-quantitatively by histologic indices. Since the biochemically defined fibrosis is a linear function of the concentration of ozone that the rats breathe, we can linearly extrapolate from our data to determine "threshold levels" of ozone that cause elevations in lung collagen synthesis rate after continuous exposure for 1-3 weeks; such linear extrapolations probably overestimate "threshold levels", if indeed such "thresholds" exist at all. Interestingly enough, we find the "threshold level" in these experiments to be less than about 0.10-0.13 ppm of ozone, approximately the former ambient air quality standard (0.08 ppm) for this pollutant (the present standard is 0.12 ppm). In preliminary experiments using cynomolgus monkeys, we have found them to be, if anything, more sensitive than rats to ozone by these criteria. Obviously most humans are not rats, nor do they breathe ozone continuously at these levels for weeks at a time. The relationship of such "threshold" values obtained in experiments of this type to the setting of air quality standards is a matter of judgement.

An obvious question that comes to mind from experiments of this type is what relationship, if any, exists between data on elevations in lung collagen synthesis rate and changes in lung collagen content, especially over a long-term exposure regimen. We have attempted to answer this question by analyzing lung collagen synthesis rates and total lung collagen content in rats chronically exposed to 0.5 ppm of ozone for up to six months (Last and Greenberg, 1979). Lung collagen synthesis rates were elevated above control values at all times studied (3, 30, 50, 90 and 180 days of continuous exposure). Lung collagen content (estimated as mg of hydroxyproline per lung) was significantly greater in the exposed rats for up to 3 months of exposure. Thus, elevated lung collagen synthesis rates seemed to be associated with elevated lung collagen content in these rats. These changes could be correlated with mild centriacinar fibrosis observed in other rats from the same study (Boorman et al., 1980).

We have also been able to show (Reiser and Last, In Press) that elevations in lung collagen synthesis rate in rats exposed to high levels of ozone (1.5 ppm for one week) are associated with a qualitative shift in the types of collagen being made and deposited in their lungs. In rat lungs the normal ratio of collagen types being synthesized is about 66% Type I collagen and 33% Type III. In the rats exposed to high levels of ozone the ratio of collagen types being synthesized is about 80-85% Type I and 15-20% Type III. It is certainly relevant to point out that Type I collagen is stiffer and less compliant than Type III; it is also what pathologists call collagen, as opposed to Type III collagen, which may be what is called reticulin. Presumably such long-term structural changes are also occurring in the lungs of our rats exposed to 0.5 ppm of ozone for several months, although we have not as yet tested this possibility experimentally. Such an experiment is currently being performed in monkeys exposed for one year to 0.8 ppm of ozone. I would again like to point out that the collagen synthesis rates by lung minces from rats exposed to ozone for up to six months remain elevated over control values; as evaluated by this parameter the rats do not show a decreased response to continued ozone exposure. There are long-term structural changes occurring in these lungs from chronically exposed rats that make them different than "normal" lungs from control animals; whether these

structural changes are considered to be good, bad, or neutral for these rats presumably depends more on one's philosophical beliefs than on any rigorous scientific comprehension of the relationship of such structural changes in the lungs to their functional capacity.

Thus, at the risk of being mildly controversial, I'd like to ask whether there is a scientific basis for the entire concept of "adaptation". I'd further like to suggest the analogy between "adaptation" and the fable of the Emperor's New Clothes. Like the minions viewing the Emperor, each discipline seems to see different clothes on this August Being; however, each describes this varied wardrobe with the same word! For example, "adaptation" to ozone according to some physiologists means that exposure-induced bronchoconstriction in human subjects at days one and two is more severe than at day four; perhaps this experiment merely titrates the ability of cells to secrete histamine under repeated stress? To some morphologists, "adaptation" to ozone means that early cellular changes in the lung decrease in extent or stay constant in the face of continuing exposure; perhaps this experiment merely defines the ability of the test animals to maintain an inflammatory influx of macrophages and/or leukocytes at sites of injury. To some biochemists, "adaptation" to ozone means that increased levels of enzymes, cofactors, total proteins, and just about anything else that can be quantitated in lung homogenates become decreased toward control values in the face of continuing exposure; perhaps this experiment merely provides biochemical footprints of the cellular (and acellular) components entering the lung during the inflammatory response and its subsequent attenuation.

I think the data I have presented in this paper suggest that when we assay for parameters related to lung structure rather than to lung inflammation, we do not observe any "adaptation" occurring with continued insult for several months of exposure. Perhaps the time is ripe to either redefine "adaptation" to allow its use only as being legitimate for one discipline (all others to select synonyms, perhaps?), or to question whether the concept still has heuristic value or biological significance. To return for the last time to our sartorially resplendent Emperor, perhaps the next generation of experiments on potential health effects of exposure to oxidant air pollutants could profit from attempts to correlate studies not of "adaptation" without rigorous definition of the term, but rather to attempt to define the basis of lung pathophysiology in terms of well described shirts (morphology), trousers (biochemistry), hats (physiology), and shoes (microbiology).

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## OPEN FORUM

DR. CROCKER (University of California, Irvine): The panel at this point will initiate the Open Forum. I would like to introduce a panel member from Battelle Northwest who is joining us for the Forum and who has some additional material to deliver as original and separate information. This is Dr. Paul Mihalko, who has been with Battelle Northwest for the last year, having completed his graduate training just prior to that. He has been developing pulmonary function analyses at Batelle Northwest. It would be timely to have him present his information at this moment. Dr. Mihalko will discuss the exposure of rats to cigarette smoke over a long-term period.

DR. MILHALKO (Batelle Northwest Laboratories): Thank you, Dr. Crocker. The complete report of the study I shall present is available from Enviro Control, Incorporated. The report number is SHP75121 and it was completed in 1979. Essentially, it was a two-year study in which Fischer 344 rats were caused to inhale cigarette smoke from three different types of cigarettes which had combinations of high tar and low nicotine, high tar and high nicotine, and low tar with low nicotine. Without presenting another series of slides this afternoon, of which we've had enough, there were just a few observations from this study that were particularly interesting with respect to the data reported earlier today. We observed some changes in basic respiratory variables that can be correlated with some of the previously presented information. It seems as if there is a speeding up of the aging process. Tidal volume increased in the smoking rats as compared to sham controls during the exposure although the sham controls caught up with the smoke exposure animals after approximately eight more months of exposure. We interpret that finding as an accentuation of a particular process for which we do not have the data to further evaluate. In any case, I thought it was particularly interesting, in light of a lot of the biochemical, morphologic, and other studies presented today that we did notice those trends.

DR. CROCKER: This will be the time for anyone from the audience to ask questions of members of the panel or for members of the panel to exchange questions among themselves.

DR. TORKELOSON (Dow Chemical Company): Dr. Jackson, you presented a slide with a curve that had resistance plotted on the vertical axis and frequency on the horizontal that I couldn't understand. I can understand increase of resistance with increased frequency of breathing, but was that frequency value breathing rate or frequency on the oscillating speaker?

DR. JACKSON (University of California, Davis): That was the frequency on the oscillating speaker. These measurements are all done under forced oscillatory conditions. In the rat study, the rats were allowed to breathe against the closed chamber and the chamber was large compared to their tidal volumes so there wasn't much breathing pressure.

DR. COHEN (Aerospace Corporation): Dr. Jackson, I would like to know what made you choose the frequency region around 100 cycles for your probe. I don't really know how acoustic energy is transferred from large tubes to small tubes, but I would imagine that it's going to be very non-linear, especially as the wavelength approaches the diameter of the various orifices. Why did you

choose that frequency region? Did you expect a linear or non-linear response as you changed the frequency from the kilocycle to megacycle range?

DR. JACKSON (University of California, Davis): There are two regions of frequencies that you can work in. One is the region where you can assume that the system behaves like a system of lumped parameters. This will be in a frequency region where the wavelength of the excitatory signal is long compared with the dimensions of the airways. In a human, taking mean airway path length of something like 40 centimeters and assuming that waves propagate at the speed of sound in free air, you can use a lumped parameter type analysis up to about 200 hertz. In the rat, where the dimensions of the airways are much smaller, you can go to a much higher frequency still using the lumped parameter system to explain your results. If you're working in that domain, or in the low frequency domain, what you would like to do is use frequencies well below or well above the resonant frequency. This gives you the best estimation of compliance and inertness. By trial and error, I was able to find the resonant frequency of a rat, which is around 42 hertz. I went down in frequency, started at 20 hertz and tried to go up to 128 hertz but I rarely get good information at a frequency that high.

DR. COHEN: In other words, you're finding that resonance is the length of the column.

DR. JACKSON: No, we're still working in an area where the system is a lumped parameter system. So dimensions of the lung are meaningless. We don't have to take dimensions into account, so we do not have resonance as a standing wave that is in the airways. We have what is generally termed a Helmholtz resonator where you can lump all of the inertness of the gas into one inert element and all of the compliance elements into a single compliance factor.

DR. COHEN: Thank you.

DR. CROCKER (University of California, Irvine): As you found some effects in animals exposed for three days, you concluded that there could no longer be the assumption of a unit inertness or a unit compliance. Was this because you supposed there to be now a new morphologic character to the air column?

DR. JACKSON (University of California, Davis): Basically there are two models we could investigate and that have been proposed in the literature. One was proposed in 1965 by Jerry Mead, who claimed that if peripheral airway resistance was high enough we would have another pathway by which flow can be shunted from going into the lung by expanding the airway wall itself. You would then set up vibrations in the airway wall. This model has been used extensively for dogs and humans and in using this model, Mead was able to separate upper and lower airway resistance components. This might be the appropriate model to use on these rats. There is some justification for using this model in that we would expect peripheral airway resistance to be elevated. The other model that we could investigate would be one where you have parallel branches within the airways so that we could lump all of the normal airways into one branch that has one RLC component and all abnormal airways into a second branch where it has a separate and different RLC component. This model, as

well, would mimic the kind of experimental data that we have. I don't know yet which one is the best.

DR. MACH (The Aerospace Corporation): A question was asked earlier about the effects of acute high-level exposure to oxidizers, and particularly NO<sub>2</sub>, and the answer given stated there was no real lasting effect of this. However, in the last three papers I heard this afternoon it seems that there were lasting effects. In fact, the effects of these acute exposures seem to increase with time. The investigators saw an effect on lavage fluid and they saw effects in the SEM pictures. Is there or isn't there an effect?

DR. LAST (University of California, Davis): I think the earlier question was asked about a single exposure to a high atmospheric NO<sub>2</sub> concentration level and I think all of the data presented in those last three papers were related to continuing exposure to oxidants.

DR. MACH: It's true that the later exposures weren't single pulse exposures, but even after those high level two to four week exposure periods were over, there were continuing effects on the lungs. Would not one high-level exposure also cause these effects? Is there that much difference between these kinds of exposures?

DR. LAST: I think we're in one of those grey areas where when you look with different techniques, you have the potential to see different things. The exposure of a human to a high concentration of NO<sub>2</sub> obviously has constraints on what can be measured. Most of the pulmonary function testing that might be done on a healthy human wouldn't reach the kinds of changes I look at. They probably wouldn't show the kinds of changes that Dr. Schwartz finds and certainly would not measure the sophisticated kinds of changes that Dr. Jackson is looking at.

DR. FREEMAN (SRI International): I was responsible for the answer to that earlier question and I think the reason has been expressed already. You have to state precisely how high an exposure level, what species are used, how long an exposure period, and what toxic material you're talking about.

DR. MASTROMATTEO (Inco, Incorporated): I'd like to follow up on that question and ask whether some of the biochemical parameters that we used in experimental animals could be used in clinical medicine? Could we not use cytologic method on sputum and bronchial washings in cases that have acute single exposures and follow them through the recovery phase?

DR. HENDERSON (Inhalation Toxicology Research Institute): I think segmental bronchial washings are already used in humans to determine neoplastic changes and to look for cytologic changes. Dr. Kilstra at Duke has done lavages in people for therapeutic measures. I think there is a potential for using segmental bronchial washings in humans. We developed the method as a screening exchange in experimental animals. You wouldn't want to do a total lung lavage in a human to see if they had been damaged by acute exposure, but there might be cases where segmental washings would be pertinent.

DR. CAVENDER (ToxiGenics, Inc.): Dr. Last, there is some indication that in the lavage fluid the divalent cations used are very important as to what you get in terms of cell numbers and perhaps even distribution of cells. The question you raised about adaptation is of particular interest. I wonder if you might speculate on the fact that your Type I collagen versus your Type III collagen changes with adaptation. Is that change in the right direction that would be predictive of emphysemic changes in the terms of losses of alveolar septa? How do you look at some of these changes that seem to be passive or that seem to disappear such as increased mucin production? Could these not be defense mechanisms that may disappear with time and therefore the adaptive process disappears? Is it possible that what you measured was not a true toxic response in any sense?

DR. LAST (University of California, Davis): The exposure in the experiments I presented was to ozone so the chronic disease associated with it would be fibrosis and the changes in collagen types we're finding are consistent with what is thought currently to be associated with pulmonary fibrosis, that is, a shift to more Type I collagen. We have not looked at this shift in an emphysematous model. We are planning some studies with NO<sub>2</sub>. Dr. Freeman has shown that NO<sub>2</sub> causes fibrotic as well as emphysematous changes so there may well be ambiguity there. I deliberately raised the point about adaptation in my talk because the concept of adaptation sort of transcends science to philosophy. What we're doing is putting a value judgement on changes that are being measured. I am perfectly comfortable with either adaptive changes that are taking place or, conversely, changes that have no value or are negative to the organisms that are transient. One of the common things that occurs with exposure to all of the noxious agents is pulmonary edema, with or without an intra or interstitial cellular inflammatory response. I think we can explain all of these changes as being related to the edema and/or the inflammation. Certainly, for example, changes in sialic acid content in a lavage report can equally be interpreted as being from membrane fragments because many of the glyco-proteins of the cell membrane contain sialic acid as does mucin. I think we fall into the trap of saying, "If I were Mother Nature designing the system to protect itself, what would I do?". We really don't understand the meaning of the changes but we tend to interpret them as being good based on the human physiology. I don't know that we really can separate cause and effect yet based on biochemical measurements.

DR. FREEMAN (SRI International): We recently received some data on our rats from Jerry Sayer at the University of Tennessee. In rats continuously exposed to NO<sub>2</sub> at 15 ppm for one year, there was about a 20% elevation in total collagen and a reduction in Type III of about 14%.

LT. COL. MELLICK (Letterman Army Institute of Research): I have a question that might be addressed by three of the panelists, Dr. Henderson, Dr. Schwartz, and Dr. Last. Both Dr. Henderson and Dr. Schwartz indicated that they had difficulty in recovering cellular elements by lavage from animals after several weeks in exposure. Dr. Last indicated that at approximately the same time in the exposure there were changes in the characteristics of the glycoprotein secretion. It seems that heavily sulfated glycoproteins are more viscous. Do you think this might be because these cells are trapped there by the mucus secretion? In Dr. Schwartz's micrographs, it appears that cells are still present but he's not able to recover them.



DR. LAST (University of California, Davis): I think it's really speculative. There are probably a number of reasons you could use to explain why you don't get the cells out. One possibility is that the airways are more sensitive and the lavage fluid doesn't go in and out as easily after prolonged exposure to irritants. Thus, airway sensitivity may be one possibility. Another possibility is that fibrin may be trapping the cells and there may be changes in cell secretion. There's probably a variety of reasons that may help explain why you don't get as many cells in lavage samples from the chronically exposed lung.

LT. COL. MELLICK: Since this technique has some promise to help in evaluating animal response without killing them, is it worthwhile to explore the possibility of injection of another substance with the lavage in order to increase the cellular yield?

DR. LAST: I really don't know. You could try it, but then you're faced with the difficulty of how long to leave this material in the lung before withdrawing it. If you were to use something like trypsin to try to get different things out, I think you'd probably be introducing more variables than you'd want. It's a problem you have to live with.

DR. HENDERSON (Inhalation Toxicology Research Institute): In our assay, when I had difficulty in getting cells out, if you looked at the morphology, it wasn't surprising that we weren't washing out many cells because it was obvious that many of the airways were blocked. In that instance, you are beyond the stage where lavage should be used. Usually by the time the animal has that much lung injury, it's panting and you don't have to lavage it. I see lavage as useful at a lower level of injury where you don't have obliteration of airways and I would recommend it for that area of screening rather than looking at severely injured animals.

DR. LOCK (Oak Ridge National Laboratory): I'd like to ask Dr. Schwartz two questions. First, you showed a slide in which you had alveolar macrophages and carbon coated latex spheres associated with those macrophages. Have you any method for determining whether those particles are actually within the macrophages being engulfed by the macrophages, or just sitting on top having no direct relationship to those cells? Second, have you ever done any work with the bacteriocidal activity of alveolar macrophages following exposure to ozone?

DR. SCHWARTZ (University of California, Davis): In response to your second question, we have looked at bacteriocidal activities in a variety of different animal species, but we have not specifically focused on in vitro bacterial killing after ozone exposure. Dr. Goldstein, a member of our group, has worked with the infectivity model in bacteriocidal activity in vivo, and there are indications that bacteriocidal activity is suppressed in that situation. The first question was about phagocytosis. There are methods available in which you allow the phagocytosis assay to go to completion and then dissolve the latex beads using a solvent such as xylene which leaves an empty vacuole inside the cell. In that situation, you know that the latex bead was inside the cell because there is now a vacuole in the cytoplasm. We have

not really concerned ourselves whether the particle was attached to the surface or was inside, because in my opinion attachment is actually the first phase of phagocytosis. If you look at the slides critically you can tell if the cytoplasmic membrane is around the latex particles because the particles don't look as distinct. The outside portion of the carbon is not a discrete line if it's actually been endocytosed. That's a different procedure that takes a lot of effort so we don't concern ourselves with whether it's attached or endocytosed because those that are attached will undoubtedly be endocytosed within a matter of minutes after contact with the particle anyway. But if you want to look at the mechanisms of phagocytosis, there are procedures for demonstrating the location of the particle and the xylene dissolving technique is one.

DR. DALBEY (Oak Ridge National Laboratory): I have a question for Dr. O'Neil. In your carbon monoxide diffusing capacity measurements, over what time period do you perform your measurements? What is the time dependency of the actual number you get?

DR. O'NEIL (U. S. Environmental Protection Agency): We use a ten second period of breath holding when we make that measurement. The calculated diffusing capacity is time dependent but I can't tell you how time dependent it is. The argument we have used when we study animals that have a disease such as emphysema as compared to control animals is that we're very careful to do the measurements with the same period of forced breath holding. A person can reproduce that breath holding technique to within a quarter of a second on a ten second period.

DR. DALBEY: Are you using GC analysis, Dr. O'Neil?

DR. O'NEIL: Yes, we analyze the alveolar sample gas on a GC. We're using 0.5% neon and 0.5% carbon monoxide for diffusing capacity measurements. That was published in the July 1980 issue of the Journal of Applied Physiology under the name of Takazawa.

MR. MOORMAN (National Institute for Occupational Safety and Health): I guss I'll direct mine to Dr. O'Neil also while he's busy there.

DR. O'NEIL (U. S. Environmental Protection Agency): All my friends are here.

MR. MOORMAN: I noticed that there has been tremendous effort expended in the development of research methods for use with rats. I also see a number of problems and I'm wondering whether people are pursuing the rat because of the economic reasons or because they feel it's a sensitive model for extrapolation with regard to human health. I noticed in the data you presented that you demonstrated a reduced total lung capacity in the rat.

DR. O'NEIL: You're correct. I included that data on oxygen because the interest was in emphysema as a model in studies of oxidant gases and presume that oxygen would be considered an oxidant gas.

MR. MOORMAN: How do you feel about the rat model? Should we be looking for better models from the comparative physiologists?

DR. O'NEIL: Yes, of course.

MR. MOORMAN: Are there any better models?

DR. O'NEIL: I think one does need to look for better models. I think the reasons for using the rat in studies of pulmonary irritants are several fold, not the least of which is the fact that they are much less expensive to use than sub-human primates. The tests are clearly easier to perform on larger animals. You don't need to go through all the concerns and worries about frequency response and miniaturizing equipment. So from the point of view of toxicity testing, I think that small animals are attractive because you can expose larger numbers and often obtain more data to increase the confidence of results. I think the reason there's been so much interest in performing pulmonary function tests in small animals is broader than that. I think there is a great deal of interest for comparative physiology purposes, for understanding how small animals respond as compared to larger animals, and, certainly, the area of extrapolation to humans is of vital interest to EPA researchers who are exposing small animals to environmental pollutants. If you're going to say that an effect is actually occurring in larger animals, you have to get good reproducible values on small animals. One of the slides I showed you was of our measurement of the diffusing capacity in a series of small animals that varied over a body mass range of about 40 to 3000 grams which included hamsters, rats, guinea pigs and rabbits. That plotted data provided a beautiful straight line with a correlation coefficient of about 0.98. There is a very high correlation between body mass and the measurement of the diffusing capacity that should extrapolate on up through larger animals, including humans.

DR. MIHALKO (Battelle Northwest Laboratories): A lot of problems in animal models have been overcome recently in the miniaturization of diagnostic techniques currently used in human pulmonary function evaluation. In one of our recent studies, as yet unpublished, we treated 60 Sprague-Dawley rats with 50 units of porcine elastase per hundred grams of body weight in saline and compared them with saline treated control rats. The results were remarkably consistent with the findings of pulmonary function changes in documented human pulmonary emphysema. I'll make that data available to you or anyone else. The volume parameters seem to be very expressive of those changes.

DR. CAVENDER (ToxiGenics, Incorporated): I'd like to ask Dr. Last one additional question. I'm very much interested in the followup to the data already presented. In particular, I was involved in an aluminum chlorohydrate study some years back and at a six month sacrifice of animals exposed to aerosol concentrations of 25 milligrams per cubic meter, the lungs were enormous. They were filled with aluminum chlorohydrate. The lymph nodes were engorged with macrophages that had engulfed the particulate material and I thought at the two year mark, this effect would be even greater. The effect was not nearly as striking at two years as it was at six months. Would

you comment on what sort of changes might take place that would lead to this type of result? In this case the granuloma did persist which may have affected the results.

DR. LAST (University of California, Davis): That's a problem area of considerable concern. There are not many animal models of long-term chronic response. We've done experiments in collaboration with Hans Peter Witschi at Oak Ridge National Laboratory who has a model of exposure to butylated hydroxytoluene and oxygen where the collagen persists out to six months and then somewhere between six months and one year, they revert back to histologic and apparent biochemical normalcy. The only material we have looked at in which the effect continues to progress across the time frame we're looking at is intratracheally instilled crystalline silica. I think that some sort of cellular memory, whatever that means in terms of lung insult, is required for these changes not to reverse. I think, from my reading of the literature and first hand experience, that silica may be unique in being ingested, released and continually cycled without really leaving the lung, thus continuing to be a primary insult. In the case of the aluminum chlorohydrate, the relationship between granuloma and fibrosis is, in human clinical medicine, kind of tricky. We're talking about a compound that is to some extent soluble and may ultimately be cleared from the lung.

DR. CAVENDER: I'd like to add that what you're possibly seeing from a pathologist's standpoint is that some of the body defense systems are successful in some instances and with aluminum chlorohydrate it may well be that the macrophage population is slowly increased and is successful in scavenging the particulates that are there. Increased numbers of phagocytes may have been successful in isolating and locating the particulate material which may cause the lesion to remain at status quo with no further increase in magnitude.

DR. CROCKER (University of California, Irvine): I'd like to ask a question that relates to that same point. Dr. Henderson noted that there was a continuation of protease activity in the tissue of the lung 8 to 10 months after the cessation of a two month exposure and in the report of Hyde, Orthoefer, Dungworth, Tyler, Carter and Lum on morphometric and morphologic evaluation of pulmonary lesions in beagle dogs there appeared to be a delayed chronic response of emphysematous-like disease. Between the ending of an exposure and the findings taken at that time and two years later in the case of the beagle dog and in the case of the rats described by Dr. Henderson, an 8-10 month period where in the absence of any further exposure there appears to have been continuation of the pulmonary response. What is the basis for this? This doesn't appear to be a case where there are residual particles trapped in the lung, for example.

DR. LAST (University of California, Davis): I think a good individual to answer that would be one of the authors of the paper who is present.

DR. DUNGWORTH (University of California, Davis): The answer is that we do not know what causes the response to continue. There are a number of these continuing processes that are documented. Another is the elastase emphysema model in which there are immediate changes in elastin within the

first week or two after the admission of the porcine elastase but, there is also a continuing remodeling of the elastic fibers for some time after. Another group has also reported a continuing change in the bronchiole epithelium using the elastase model. In the study you mentioned we just don't know why those changes occurred. One of the features of the damage was a micro-nodular proliferation of Clara cells which persisted in spite of the absence of the irritant which had produced them in the first instance. Presumably, these cell populations once they're triggered to a certain stage of persistent hyperplasia, have a continuation of that same hyperplasia in the daughter cells. In other words, it's almost as though there is genetic damage. If that seems to be too unlikely, given the nature of the stimulus, then maybe like many organs the lung reaches a stage wherein the damage is self-perpetuating and in some instances progressive, such as occurs in the liver. There may be sufficient damage to the epithelium of the small airways that clearance is affected and there is not the same protection against the constant onslaught of inhaled particles as would normally occur and in these damaged lungs it may be sufficient to cause a slight progressive lesion. These are speculative thoughts but there are obvious prolonged or delayed responses in these several models.

POSTER SESSION

Chairman

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THE ACUTE INHALATION TOXICITY OF DIESEL FUELS USED IN  
VEHICLE ENGINE EXHAUST SMOKE SYSTEMS (VEESS)

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INTRODUCTION

Toxicity studies were conducted to determine acute inhalation effects from single exposures to two types of diesel fuel smokes and/or exhaust generated by an M60A1 tank. These tests were conducted in support of the Vehicle Engine Exhaust Smoke System (VEESS) experimental program and the purpose for selected experimental procedures was to produce exposure conditions that are similar to those encountered in simulated tactical exercises by unmasked personnel. The reason for using the M60A1 tank to generate the smoke and/or exhaust was because no other method of dissemination was available to furnish comparable atmospheres of the combination of the engine exhaust products and oil smoke produced by volatilization/condensation in and on the engine manifold.

The fuels used in these tests were Diesel Fuel #2 (DF2) and #1 (DF1) which are used in the VEESS systems as Summer and Winter grade fuels, respectively. The attempted concentrations of generated smokes for both DF2 and DF1 were 35 mg/L (35,000 mg/M<sup>3</sup>) which are shown in Figure 1 to be the theoretical peak smoke concentration predicted for exposure of personnel standing up to 10 meters downwind from the tank, operating at maximal smoke generating efficiency under strong inversion and low wind speed atmospheric conditions. Since dissemination of M60A1 tank generated smoke also involves normal exhaust emissions from internal engine combustion, the concentration of this component was also monitored and evaluated for toxicologic effects: Total hydrocarbon concentration of the exhaust for either type of fuel was found to be 0.4 - 0.6% of the smoke concentrations.

In general, the toxicity studies were performed by exposing 2-3 species of animals to M60A1 tank generated DF2 and DF1 smoke and/or vapor in a 20,000 liter chamber under static air flow conditions. Exposure periods ranged from 15-300 minutes. Toxicologic, physiologic, hematologic, blood chemical and pathologic assessments were made over a 14 day post-exposure period.

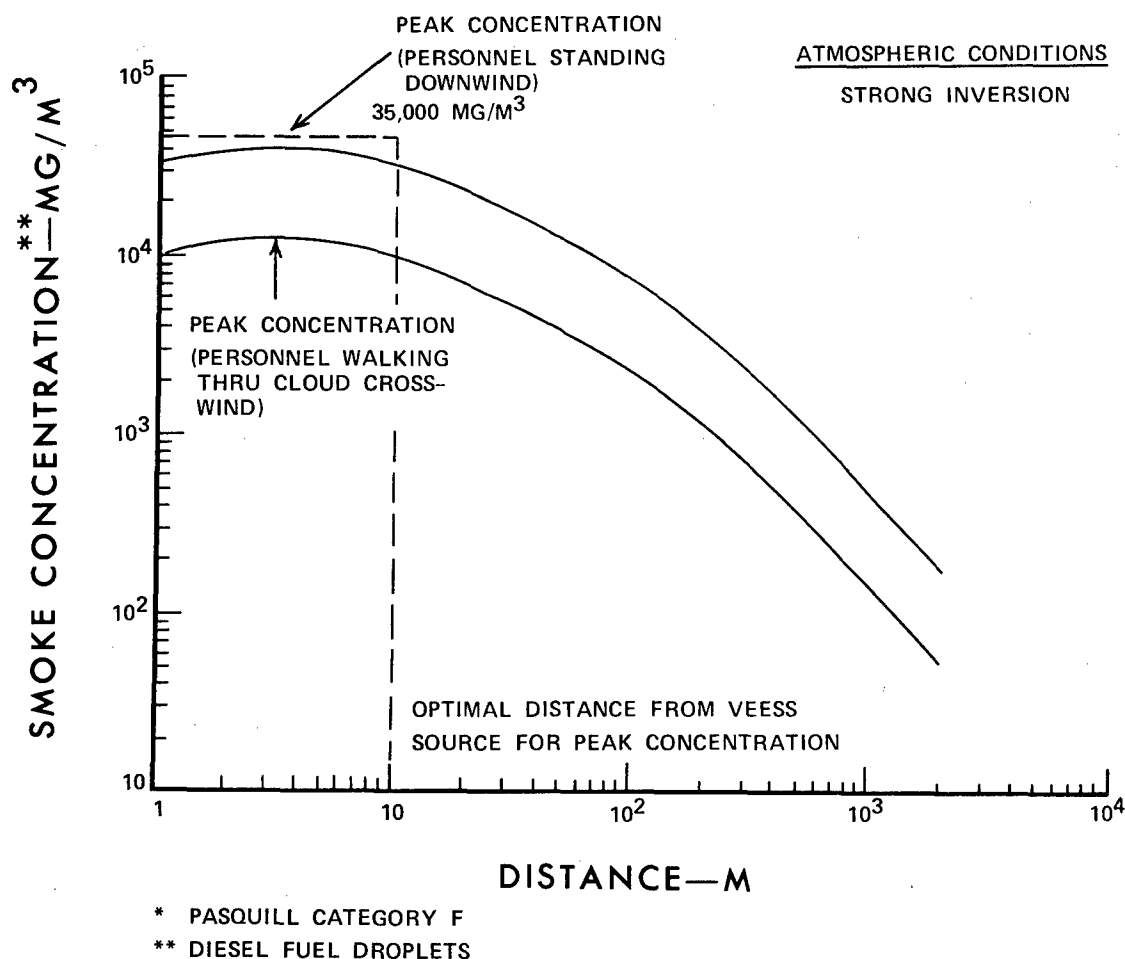


Figure 1. The optimal diesel fuel smoke generation capacity of a Vehicle Engine Exhaust Smoke System (VEESS) operating under inverse atmospheric conditions\*.

### EXPERIMENTAL PROCEDURES

#### M60A1 TANK DF2 AND DF1 SMOKE AND/OR EXHAUST GENERATION

Tank generation and dissemination of the diesel fuel smokes and/or exhausts consisted of accelerating the 750 HP Chrysler engine up to 1700 rpm's for approximately 5 minutes. During this acceleration period the tank was kept in a stationary position with no load on the engine. When the engine manifold temperature reached 1180°F the selected fuel was expelled into it through a stainless steel orifice at a rate of 1,720 L/min.

The generated smoke/exhaust was then drawn through a six inch flexible stainless steel tube attached to the tank manifold into a wind tunnel operating at 3 miles per hour. After a generation period of 5 minutes, the smoke/exhaust was shunted into a 20,000 liter cylindrical exposure chamber by means of a series of gate valves. The smoke/exhaust was generated into the exposure chamber for 50 seconds at an air flow rate of 4,000 liters per minute to attain



target concentrations. The chamber was then sealed and the cloud trapped in the exposure section. The dilution ratio of the smoke/exhaust to air was 1-3. Caged animals were placed in the chamber prior to the introduction of the smoke. At the end of the designated exposure period, chamber valves were opened and the smoke/exhaust evacuated over a 10 minute period. This procedure was followed for 15-minute and 60-minute exposures. For exposures exceeding 60 minutes, the total procedure was repeated in 60-minute increments because the smoke/exhaust concentration over a single 1-hour exposure was found to degrade as much as 65%. The same procedure was followed for the exhaust alone exposure omitting expulsion of fuel into the hot engine manifold.

During a 60-minute exposure, ambient and chamber temperatures and relative humidity were measured with a Honeywell Instrument Company analyzer Model #W809A, before and at 1, 5, 10, 20, 30, 40, 50 and 60 minutes after smoke and/or exhaust entry into the chamber.

Total hydrocarbon content, carbon monoxide, and carbon dioxide concentrations were measured at the same intervals as temperature and relative humidity as compared to 0, 10, and 60 minutes for determination of oxygen content. If the exposures exceeded 60 minutes, the same time intervals for measuring the above parameters were followed. In the case of 15-minute exposures, similar readings were taken excepting the final reading was recorded at 15 minutes.

Total hydrocarbon content and carbon dioxide were measured on a Horiba-LTD infra red analyzer MEXA-200. Carbon monoxide was measured with a Beckman Model #215B analyzer. Chamber oxygen content was obtained by measuring aliquots from grab samples on a Beckman analyzer Model E2.

Several single measurements for sulfur dioxide, nitrogen dioxide, and ethylene oxide were taken throughout the experiments with either fuel to determine the chamber concentration of these gases in smoke and/or exhaust. These measurements were made with Draeger tubes, usually 10 minutes after smoke and/or exhaust entry.

For DF2 and DF1 smoke/exhaust, chamber samples were drawn through a 350 ml glass cylinder "grab sampler" at a flow rate of 5 liters per minute for 1 minute, then trapped using pet cocks at the intake and exhaust ports of the cylinder. Collection times were at 10, 30, and 59 minutes each 60-minute exposure segment. For 15-minute exposures, collection times were at 9 and 14 minutes. The glass cylinders were then immersed in an isopropyl alcohol/dry ice bath at -70°C for 30 minutes. Ten milliliters of hexane was next added to the cylinders and shaken vigorously, then 1-5 microliter aliquots extracted for gas chromatographic analysis. Analyses were performed using a Hewlett-Packard Gas Chromatograph Model 5830A with a glass column containing 10% OV-1 Chromosorb W, Mesh 80-100. Column temperatures were programmed from 110° to 260° C. A Hewlett-Packard Model #18850A terminal was used. Standard solutions of diesel stock aliquots in the same concentration ranges were simultaneously analyzed for baselines.

For chamber sampling of DF2 exhaust total hydrocarbon content, a modified Mine Safety Appliance Company Electrostatic Precipitator (Model no. series F) was used. Sampling rate was 1 liter per minute for 10 minutes and samples were taken at 1, 20, and 50 minutes for each exposure hour. For 15 minute exposures, DF2 exhaust samples were taken from 5-15 minutes.

With DF1 exhaust, both aerosol and vapor were disseminated so the aerosol was collected with a precipitator and the vapor trapped in a Spherocharb (activated charcoal) collection tube in tandem with the precipitator. Sampling rate was 5 L/min for 5 minutes and samples were taken at 5, 30, and 55 minutes for each exposure hour. For 15 minute exposures, DF1 samples were taken from 1-6 minutes and 10-15 minutes.

DF2 and DF1 exhaust hydrocarbon aerosol was extracted from the precipitators with 15 ml of hexane. DF1 exhaust vapor was extracted from the Spherocharb tubes with 10 ml of hexane and comprised 25% of the total hydrocarbon collected.

A detailed description of the methods for analyzing DF2 and DF1 smoke and/or exhaust hydrocarbon particles and vapor are shown in Tables 1 and 2.

TABLE 1. CHEMICAL PROCEDURE FOR DETERMINING THE TOTAL HYDROCARBON CONTENT OF M60A1 TANK GENERATED DF2 SMOKE AND/OR EXHAUST

<u>METHOD</u>	<u>SMOKE / EXHAUST</u>	<u>EXHAUST</u>
1. <u>ATTEMPTED AIRBORNE CONCENTRATION (Mg/L)</u>	35	0.2
2. <u>SAMPLER</u>	350ML GRAB SAMPLER	MINE SAFETY APPLIANCE CO., ELECTROSTATIC PRECIPITATOR (MODEL NO. SERIES F).
3. <u>SAMPLE COLLECTION TIME (SEC)</u>	50	50
4. <u>AIR TO HYDROCARBON MIXTURE DILUTION</u>	3:1	3:1
5. <u>SAMPLE COLLECTION FLOW RATE (L/Min.)</u>	5	1
6. <u>SAMPLING TIME (MIN)</u>	1	10
7. <u>SAMPLING PERIODS (MIN)</u>	15 (9-10, 14-15) 60 (10-11, 30-31, 59-60)	15 (5-15) 60 (1-11, 20-30, 50-60)
8. <u>CHEMICAL ANALYSIS</u>	BATHE GRAB SAMPLER IN ISOPROPYL ALCOHOL/DRY ICE BATH FOR 30 MIN AT -70°C. ADD 10ML OF HEXANE. ANALYZE (1-5 µl) ALIQUOTS ON HEWLETT PACKARD GC MODEL No. 5830A GLASS COLUMN- 10% OV-1 CHROMOSORB W, MESH 80-100. USE HEWLETT PACKARD GC TERMINAL NO. 18850A	WASH PRECIPITATOR CANISTER CONTENTS WITH 15ML OF HEXANE. ANALYZE (1-5 µl) ALIQUOTS AS DESCRIBED FOR SMOKE/EXHAUST MIXTURES

TABLE 2. CHEMICAL PROCEDURE FOR DETERMINING THE  
TOTAL HYDROCARBON CONTENT OF M60A1 TANK GENERATED DF1  
SMOKE AND/OR EXHAUST

<u>METHOD</u>	<u>SMOKE / EXHAUST</u>	<u>EXHAUST</u>
1. <u>ATTEMPTED AIRBORNE CONCENTRATION (Mg/L)</u>	35	0.2
2. <u>SAMPLER</u>	350 ML GRAB SAMPLER	MINE SAFETY APPLIANCE CO. ELECTROSTATIC PRECIPITATOR (MODEL NO. SERIES F) IN TANDEM WITH SPHEROCARB (ACTIVATED CHARCOAL) FILTER
3. <u>SAMPLE COLLECTION TIME (SEC)</u>	50	50
4. <u>AIR TO HYDROCARBON MIXTURE DILUTION</u>	3:1	3:1
5. <u>SAMPLE COLLECTION FLOW RATE (L/Min)</u>	5	5
6. <u>SAMPLING TIME (MIN)</u>	1	5
7. <u>SAMPLING PERIODS (MIN)</u>	15 (9-10, 14-15) 60 (10-11, 30-31, 59-60)	15 (1-6, 10-15) 60 (5-10, 30-35, 55-60)
8. <u>CHEMICAL ANALYSIS</u>	BATHE GRAB SAMPLER IN ISOPROPYL ALCOHOL/DRY ICE BATH FOR 30 MIN AT -70°C. ADD 10ML OF HEXANE. ANALYZE (1.5 µl) ALIQUOTS ON HEWLETT PACKARD GC MODEL NO. 5830A- GLASS COLUMN- 10% OV-1 CHROMOSORB W, MESH 80-100. USE HEWLETT PACKARD GC TERMINAL NO. 18850A.	WASH PRECIPITATOR AND SPHEROCARB CONTENTS WITH 15ML AND 10 ML OF HEXANE RESPECTIVELY. ANALYZE (1.5 µl) ALIQUOTS FROM BOTH SAMPLES SEPARATELY AS DESCRIBED FOR SMOKE/EXHAUST MIXTURES

## INHALATION TOXICITY STUDIES

In the DF2 smoke and/or exhaust studies, young mature adult Sprague-Dawley rats, Fischer 344 rats, Hartley albino guinea pigs and B6C3F1 mice were exposed in groups of five males and five females each. In the DF1 studies the Fischer rats and mice were omitted. In both studies an equal number of controls were retained for each exposure and underwent the same procedure excepting exposure to the smoke/exhaust or exhaust clouds. All animals were weighed prior to exposure and at 24 hours, 1 week and 2 weeks post-exposure.

In both DF2 and DF1 smoke and/or exhaust studies, for the 15 minute and 1 hour exposures, groups of three males and three females each of Sprague-Dawley rats, Fischer rats and guinea pigs received plethysmographic measurements prior to exposure and at 24 hours and 14 days post-exposure to the DF2 smoke/exhaust and exhaust only. In the case of the DF1 exposures, Fischer rats were omitted. An equal number of controls were evaluated.

All exposed animals were observed for toxic signs and mortality immediately after exposure to the smoke and/or exhaust and daily thereafter for a 14 day period.

At completion of the postexposure period, selected exposure and control animals (usually six each) were anesthetized intraperitoneally with sodium pentathol, bled and sacrificed for gross and histopathology. Representative animals that died spontaneously during the 14 day postexposure holding period were also submitted for gross and histopathology. Blood samples were analyzed for triglycerides, cholesterol, glucose, blood urea nitrogen, creatinine, uric acid, sodium, potassium, chloride, carbon dioxide, alkaline phosphatase, serum glutamic pyruvic transaminase, serum oxalacetic transaminase, albumin, globulin, albumin globulin ratio, calcium, phosphorus, red blood cell, white blood cell, differential white cell count, hemoglobin and hematocrit. For gross and histopathology, the following organs were examined: heart, liver, lungs, spleen, kidneys, brain, eyes, trachea, nares, adrenals, stomach, skin, bladder, gonads, pancreas, small and large intestines.

## RESULTS

### CHAMBER CONDITIONS FOR INHALATION STUDIES

Both DF2 and DF1 generated smoke/exhaust clouds were extremely dense, acrid in odor and produced oily droplets on chamber equipment and animal fur. At least ninety-eight percent of the airborne particulates in both type clouds were found to be < 0.1 microns in mass mean diameter. A typical example of particulate size measurement for DF2 smoke/exhaust is shown in Table 3.

TABLE 3. THE AERODYNAMIC MASS DIAMETER OF HYDROCARBON PARTICLES COLLECTED FROM A DF2 SMOKE/EXHAUST EXPOSURE CHAMBER CLOUD

<u>IMPACTOR</u>	<u>SIZE</u> ( $\mu$ )	<u>MASS *</u> (MICROGRAMS)	<u>%</u>	<u>CUM %</u>
1	16	0	---	---
2	10	0	---	---
3	4	0	---	---
4	2.4	0	---	---
5	1.5	32	---	---
6	0.85	100	10.8	100.0
7	0.47	197	21.2	85.7
8	0.29	343	37.0	64.5
9	0.10	142	15.3	27.5
10	---	69	7.4	12.2
<u>FILTER</u>	---	45	4.8	4.8

$$\frac{\text{TOTAL MASS } 928 \mu\text{g}}{\text{TOTAL LITERS COLLECTED } 59.5} = 15.6 \mu\text{g/liter}$$

AVE. HYDROCARBON CONCENTRATION OF 20,000 LITER/EXPOSURE CHAMBER DF<sub>2</sub> SMOKE/EXHAUST CLOUD = 46.0 Mg/L

$$\frac{15.6 \mu\text{g/liter}}{46,000 \mu\text{g/liter}} = 0.04\% \text{ OF PARTICLES GREATER THAN } 0.1 \mu$$

\* AVE. OF FOUR SIERRA (2210-K) CASCADE IMPACTOR COLLECTION SAMPLES

Airborne concentrations for the smoke/exhaust mixtures for both DF2 and DF1 were based on total hydrocarbon content. For animal exposures with DF2, concentrations ranged from 33,800 to 54,000 mg/m<sup>3</sup> with a mean of 45,600 mg/m<sup>3</sup> ± 18,100. Exposure times were from 15-300 minutes. With DF1 smoke/exhaust exposures, concentrations ranged from 3,500 to 68,250 mg/m<sup>3</sup> with a mean of 42,000 mg/m<sup>3</sup> ± 16,800. Exposure times were from 15-300 minutes.

During DF2 smoke/exhaust exposures chamber temperatures ranged from 69.0°F ± 7.0 before smoke entry; 84.0°F ± 8.0 five minutes after smoke entry and 74.0°F ± 6.0 between 5-60 minutes. The relative humidities for the same time periods were 58% ± 31, 69% ± 22 and 74% ± 23, respectively. During DF1 smoke/exhaust chamber exposures the mean temperatures were 75°F ± 4 before smoke entry; 81°F ± 3 five minutes after smoke entry and 77°F ± 3 between 5-60 minutes. For equivalent times, relative humidities were 81% ± 19; 84% ± 19 and 85% ± 20.

Temperature and relative humidity rises during DF2 and DF1 smoke/exhaust emission into the animal exposure chamber were due to the heat and moisture from the M60A1 tank engine. The temperature rise usually dissipated significantly between 3-5 minutes after smoke/exhaust entry while the relative humidity remained elevated. (Tables 4 and 5)

TABLE 4. CHAMBER CONDITIONS DURING ANIMAL EXPOSURES TO M60A1 TANK GENERATED DF2 SMOKE\* AND/OR ENGINE EXHAUST\*\*

		DF <sub>2</sub> SMOKE & EXHAUST	EXHAUST ALONE
EXHAUST MANIFOLD TEMPERATURE (°F)	BEFORE	1180	1180
	DURING	500	1180
CHAMBER TEMPERATURE (°F)	BEFORE	69 ± 7	71 ± 6
	DURING (0-5 MIN)	84 ± 8	82 ± 7
	DURING (5-60 MIN)	74 ± 6	74 ± 6
CHAMBER RELATIVE HUMIDITY (%)	BEFORE	58 ± 31	60 ± 23
	DURING (0-5 MIN)	69 ± 22	79 ± 18
	DURING (5-60 MIN)	74 ± 23	77 ± 19
CARBON MONOXIDE (PPM)		68 ± 25	80 ± 19
CARBON DIOXIDE (PPM)		6763 ± 3072	7710 ± 3153
OXYGEN (%)		20 ± 0.8	20 ± 0.4

\* TOTAL HYDROCARBON CONCENTRATION OF DF<sub>2</sub> SMOKE/EXHAUST

\*\* TOTAL HYDROCARBON CONCENTRATION OF DF<sub>2</sub> ENGINE EXHAUST

\* GRAB SAMPLES

45.6 ± 18.1 MG/L  
(45,600 ± 18,100 MG/M<sup>3</sup>)

\*\* ELECTROSTATIC PRECIPITATOR

0.280 ± 0.200 MG/L  
(280 ± 200 MG/M<sup>3</sup>)

TABLE 5. CHAMBER CONDITIONS DURING ANIMAL EXPOSURES TO M60A1 TANK GENERATED DF1 SMOKE\* AND/OR ENGINE EXHAUST\*\*

EXHAUST MANIFOLD TEMPERATURE (°F)	DF <sub>1</sub> SMOKE & EXHAUST		EXHAUST ALONE
	BEFORE	1180	1180
	DURING	500	1180
CHAMBER TEMPERATURE (°F)	BEFORE	75 ± 4	75 ± 8
	DURING (0-5 MIN)	81 ± 3	83 ± 6
	DURING (5-60 MIN)	77 ± 3	78 ± 7
CHAMBER RELATIVE HUMIDITY (%)	BEFORE	81 ± 19	81 ± 15
	DURING (0-5 MIN)	84 ± 19	89 ± 8
	DURING (5-60 MIN)	85 ± 20	96 ± 6
CARBON MONOXIDE (PPM)		63 ± 11	70 ± 14
CARBON DIOXIDE (PPM)		5000 ± 9021	5379 ± 2128
OXYGEN (%)		20 ± 0.5	20 ± 0.6

\* TOTAL HYDROCARBON CONCENTRATION OF DF<sub>1</sub> SMOKE/EXHAUST

TOTAL HYDROCARBON CONCENTRATION OF DF<sub>1</sub>  
EXHAUST

\* GRAB SAMPLES

42.0 ± 16.8 MG/L

42,000 ± 16,800 MG/M<sup>3</sup>

\*\* ELECTROSTATIC PRECIPITATOR

0.142 ± 0.10 MG/L

(142.0 ± 100 MG/M<sup>3</sup>)

\*\* SPHEROCARB FILTER

0.035 ± 0.039 MG/L

(35.0 ± 39.0 MG/M<sup>3</sup>)

Chamber concentrations of carbon monoxide, carbon dioxide and oxygen taken during animal exposures to DF2 and DF1 smoke/exhaust are shown on Tables 4 and 5. Measurements for sulfur dioxide, nitrogen dioxide and ethylene oxide were also obtained and are shown in Table 6.

Airborne chamber clouds produced by M60A1 tank generated DF2 and DF1 exhaust were less dense than those of the smoke/exhaust mixtures. The odor was less acrid but oily deposits were found on the animal fur. The mass median diameter of at least 98% of the airborne particulates in both DF2 and DF1 exhaust clouds was found to be < 0.1 microns. There was a gradual degradation of total hydrocarbon concentration for DF2 and DF1 exhaust which dissipated as much as 50% by 60 minutes.

DF2 exhaust total hydrocarbon concentrations ranged from 100 to 400 mg/m<sup>3</sup>, with a mean of 280 mg/m<sup>3</sup> ± 200. Exposure times were from 15-300 minutes. Airborne hydrocarbon concentrations for DF1 exhaust (including vapor) ranged from 59 to 337 mg/m<sup>3</sup>, with a mean of 177 mg/m<sup>3</sup> ± 139. Like DF2, exposure times were from 15-300 minutes.

Mean chamber temperatures during a 60 minute animal exposure to DF2 exhaust were 71°F ± 6 before emission; 82°F ± 7 five minutes after emission and 74°F ± 6 at 5-60 minutes. For DF1 exhaust, the mean chamber temperatures were 75°F ± 8; 83°F ± 6 and 78°F ± 7 for the same periods.

TABLE 6. TYPICAL CHAMBER GAS CONCENTRATIONS\* DURING ANIMAL EXPOSURE TO M60A1 TANK GENERATED DF2 AND DF1 (DIESEL FUEL) SMOKE AND/OR EXHAUST

CHAMBER GAS MEASURED	DF <sub>2</sub> SMOKE/EXHAUST <sup>(a)</sup> (ppm)	DF <sub>2</sub> EXHAUST <sup>(b)</sup> (ppm)	DF <sub>1</sub> SMOKE/EXHAUST <sup>(c)</sup> (ppm)	DF <sub>1</sub> EXHAUST <sup>(d)</sup> (ppm)
NITROGEN DIOXIDE	20 (18-20)	17 (10-20)	35 (30-40)	35 (35-35)
SULFUR DIOXIDE	17 (10-20)	12 (5-20)	12 (5-15)	8 (6-12)
CARBON MONOXIDE	68 (35-122)	80 (50-130)	62 (28-75)	70 (45-108)
ETHYLENE OXIDE	< 0.1	< 0.1	< 0.1	< 0.1
CARBON DIOXIDE	6763 (1200-3500)	7710 (400-11,000)	5000 (3,000-6,000)	6318 (5,000-11,000)
OXYGEN (%)	20 (19.6-21.2)	20 (19.6-20.9)	20 (19.2-20.9)	20 (18.9-20.9)

**LEGEND:**

\* GAS CONCENTRATIONS ARE AVERAGE VALUES WITH ACTUAL RANGES

- (a) TOTAL HYDROCARBON CONCENTRATION OF DF<sub>2</sub> SMOKE/EXHAUST =  $45.6 \pm 18.1$  Mg/L ( $45,600 \pm 18,100$  Mg/m<sup>3</sup>)
- (b) TOTAL HYDROCARBON CONCENTRATION OF DF<sub>2</sub> EXHAUST =  $0.280 \pm 0.200$  Mg/L ( $280 \pm 200$  Mg/m<sup>3</sup>)
- (c) TOTAL HYDROCARBON CONCENTRATION OF DF<sub>1</sub> SMOKE/EXHAUST =  $42.0 \pm 16.8$  Mg/L ( $42,000 \pm 16,800$  Mg/m<sup>3</sup>)
- (d) TOTAL HYDROCARBON CONCENTRATION OF DF<sub>1</sub> EXHAUST =  $0.177 \pm 0.139$  Mg/L ( $177 \pm 139$  Mg/m<sup>3</sup>)

Mean relative humidities for DF2 exhaust were  $60\% \pm 23$  before emission;  $79\% \pm 18$  five minutes after emission and  $77\% \pm 19$ , 5-60 minutes after exhaust entry. For DF1 the mean relative humidities for the same time intervals were  $81\% \pm 15$ ;  $89\% \pm 8$  and  $96\% \pm 6$ .

As was the case with the DF2 and DF1 smoke and exhaust, both the temperature and the relative humidity rose during emission of the DF2 and DF1 exhaust clouds. This rise was due to the heat and condensation from the M60A1 tank engine. The temperature rise usually dissipated significantly about 3-5 minutes after exhaust expulsion but the relative humidities remained elevated. This is seen in Tables 4 and 5.

Carbon monoxide, carbon dioxide, and oxygen content measured during the DF2 and DF1 exhaust studies are also shown in Tables 4 and 5. Measurements for sulfur dioxide, nitrogen dioxide, and ethylene oxide content were also taken and are shown in Table 6.

### TOXIC SIGNS AND MORTALITY

During exposure to DF2 and DF1 smoke and/or exhaust, animals were not visible for observation due to the density of the smoke clouds. At the cessation of all exposure periods (e.g. 15-300 min) various toxic signs (Table 7) were noted: lethargy, apnea, hypopnea, lacrimation, piloerection, exophthalmos, cyanosis, nasal hemorrhage, frothiness of the nose or mouth, and when death was imminent tremors, convulsions, and prostration. Sprague-Dawley rats, Fischer rats, and Hartley guinea pigs showed lethargy after 15 minute exposures to DF2 smoke/exhaust. No toxic signs were shown by any of the

species after 15 minute exposures to DF2 exhaust. After 60 minute exposures to DF2 smoke/exhaust, however, several guinea pigs showed lacrimation, oral and nasal frothiness, nasal hemorrhage and tremors. Sprague-Dawley rats manifested cyanosis and mice showed piloerection. Fischer rats and guinea pigs died from this exposure. With the 60 minute DF2 exhaust exposures the only "premortem" toxic sign was lethargy in mice. Sprague-Dawley rats, mice and Fischer rats died between 6-50 hours postexposure. As exposures to DF2 smoke and/or exhaust extended beyond 60 minutes, an increased incidence of signs of lethargy and respiratory distress (e.g. hypopnea or apnea) was observed. Exophthalmos was shown by Fischer rats and Hartley guinea pigs exposed to DF2 exhaust for 120 and 180 minutes, respectively. Tremors, convulsions and prostration became more frequent as exposure to either type cloud extended beyond 60 minutes. In many instances mortalities in animals exposed to DF2 smoke/exhaust differed from those of animals exposed to DF2 exhaust alone (Table 8).

TABLE 7. TOXIC SIGNS OBSERVED IN SPRAGUE-DAWLEY RATS, FISCHER 344 RATS, B6C3F1 MICE AND HARTLEY STRAIN GUINEA PIGS EXPOSED TO M60A1 TANK GENERATED DF2 SMOKE AND/OR EXHAUST

TOXIC SIGN	DF <sub>2</sub> SMOKE/EXHAUST (Time of Exposure, Min)					DF <sub>2</sub> EXHAUST (Time of Exposure, Min)				
	15	60	120	180	300	15	60	120	180	300
CYANOSIS		SR	FR, GP					FR		
APNEA			GP							
HYPOPNEA			SR, M, FR, GP	SR, FR, GP	M, FR			M, SR		
LETHARGY	SR, FR, GP		SR, M	SR, M, FR	M, FR		M	FR, M	SR, M GP	M, GP
LACRIMATION		GP	GP							
ORAL & NASAL FROTHINESS		GP								
NASAL HEMORRHAGE		GP	GP							
PILOERECTION		M		SR, GP FR						
EXOPHTHALMOS								FR	GP	
TREMORS		GP		GP, M				FR		
CONVULSIONS								FR	GP	
PROSTRATION			GP	M, FR	SR, FR			FR		
DEATH		FR, GP	FR, GP	SR, FR, GP	SR, FR, M, GP	SR, FR, M	SR, FR, M	SR, FR, M, GP	SR, FR, M, GP	SR*, M GP

\*ALL ANIMALS EXPIRED BEFORE TOXIC SIGN OBSERVATION.

LEGEND: SR = SPRAGUE DAWLEY RAT; M = B6C3F1 MOUSE; FR = FISCHER 344 RAT; GP = HARTLEY STRAIN GUINEA PIG. (NOTE: ALL TOXIC SIGNS DISAPPEARED BY 4-24 HRS POST EXPOSURE).



TABLE 8. MORTALITY FRACTIONS DERIVED FROM STUDIES DESIGNED TO DETERMINE THE ACUTE INHALATION TOXICITY OF M60A1 TANK GENERATED DF2 SMOKE AND/OR ENGINE EXHAUST

SPECIES	M60A1 TANK GENERATED DF <sub>2</sub> SMOKE/EXHAUST					M60A1 TANK ENGINE EXHAUST				
	15	60	120	180	300	15	60	120	180	300
	EXPOSURE TIME (MIN)					EXPOSURE TIME (MIN)				
FISCHER 344 RAT	0/10	1/10	8/10	9/10	10/10	0/10	1/10	7/8	10/10	- -
SPRAGUE DAWLEY RAT	0/10	0/10	0/10	3/10	10/10	0/10	3/10	5/10	6/10	9/10
HARTLEY GUINEA PIG	0/10	4/10	9/10	5/10	10/10	0/10	0/10	0/10	5/10	9/10
B6C3F1 MOUSE	0/10	0/10	0/10	0/10	6/10	0/10	5/10	5/10	2/10	4/10
TOTAL	0/40	5/40	17/40	17/40	36/40	0/40	9/40	17/38	19/40	22/30

TOTAL HYDROCARBON CONCENTRATION OF DF<sub>2</sub> SMOKE/EXHAUST

GRAB SAMPLES

45.6 ± 18.1 MG/L

(45,600 ± 18,100 MG/M<sup>3</sup>)

TOTAL HYDROCARBON CONCENTRATION OF ENGINE EXHAUST

ELECTROSTATIC PRECIPITATOR

0.280 ± 0.200 MG/L

(280 ± 200 MG/M<sup>3</sup>)

The toxic response in these animals does not appear related to total hydrocarbon concentration since the DF2 smoke/exhaust mean concentrations were 45,600 mg/m<sup>3</sup> whereas the DF2 exhaust was 280 mg/m<sup>3</sup>.

As a result, Table 8 shows that when M60A1 tank generated DF2 smoke and/or exhaust was disseminated under the same conditions, Fischer rats were equally vulnerable to the components of both clouds; Hartley guinea pigs were more sensitive to the smoke/exhaust than to the exhaust alone; B6C3F1 mice and Sprague-Dawley rats were more sensitive to the exhaust alone. The mice appeared to be the most resistant species to both types of clouds.

Exposure of Sprague-Dawley rats and Hartley guinea pigs to DF1 smoke and/or exhaust showed essentially the same pattern of toxic signs (Table 9) and mortality as exposure to DF2. No toxic signs were seen from the 15 minute exposure to either smoke and/or exhaust except lacrimation in guinea pigs exposed to the smoke/exhaust mixture. As a result of 60 minute exposures to DF1 smoke/exhaust, cyanosis was seen in rats and lacrimation in guinea pigs. No deaths occurred. Exposure for the same duration to the exhaust caused lethargy in both species and lacrimation in guinea pigs. One rat died from the 60 minute exhaust exposure.

As was the case with DF2 smoke and/or exhaust exposures, toxic signs became more frequent and severe beyond the 60 minute exposures. Apnea was seen in both rats and guinea pigs exposed for periods beyond 60 minutes to the smoke/exhaust cloud. Lethargy was common in both species after exposure to either type of cloud. Nasal hemorrhage was observed in rats and guinea pigs after 240 and 300 minute exposures, respectively, to DF1 smoke/exhaust. The incidence of tremors, convulsions and prostration occurred with frequency after 60 minute exposure to either cloud and deaths increased.

TABLE 9. TOXIC SIGNS OBSERVED IN SPRAGUE-DAWLEY RATS AND HARTLEY STRAIN GUINEA PIGS EXPOSED TO M60A1 TANK GENERATED DF1 SMOKE AND/OR EXHAUST

TOXIC SIGN	DF <sub>1</sub> SMOKE/EXHAUST (Time of Exposure, Min)						DF <sub>1</sub> EXHAUST (Time of Exposure, Min)					
	15	60	120	180	240	300	15	60	120	180	240	300
CYANOSIS		SR			SR							
APNEA			GP	SR, GP	SR	SR, GP			SR		GP	GP
HYPOPNEA												
LETHARGY			GP	SR, GP	SR	GP	SR, GP	SR	SR	SR, GP	GP	GP
LACRIMATION	GP	GP	GP	GP		SR, GP	GP	SR		GP		GP
NASAL HEMORRHAGE					SR	GP						
PILOERECTION												
EXOPHTHALMOS						SR			SR			
TREMORS						GP						GP
CONVULSIONS				SR, GP		SR, GP						GP
PROSTRATION				GP		SR, GP				SR		
DEATH			SR* GP	SR, GP	SR, GP*	SR, GP	SR	SR, GP*		SR, GP	SR*	SR** GP

\*SPECIES NOT EXPOSED

\*\*ALL ANIMALS EXPIRED BEFORE TOXIC SIGN OBSERVATION

LEGEND: SR = SPRAGUE DAWLEY RAT; GP = HARTLEY STRAIN GUINEA PIG

(NOTE: ALL TOXIC SIGNS DISAPPEARED BY 4-24 HRS POST EXPOSURE).

Exophthalmos was seen in rats after 300 minute exposures to smoke/exhaust and 120 minute exposures to the exhaust. In many instances, mortalities in both species exposed to DF1 smoke/exhaust differed from those of animals exposed to DF1 exhaust alone.

Results from these studies (Table 10) showed that the guinea pigs were more sensitive to the DF1 smoke/exhaust than the rat. The opposite was true for DF1 exhaust alone.

As was shown with DF2, mortality does not appear to be based on total hydrocarbon concentration. DF1 smoke/exhaust mean concentration was 42,000 mg/M<sup>3</sup> and DF1 exhaust alone was 177 mg/M<sup>3</sup>. Table 10 gives the composite mortality fractions found from these studies with DF1 smoke and/or exhaust.

TABLE 10. MORTALITY FRACTIONS DERIVED FROM STUDIES DESIGNED TO DETERMINE THE ACUTE INHALATION TOXICITY OF M60A1 TANK GENERATED DF1 SMOKE AND/OR ENGINE EXHAUST

SPECIES	M60A1 TANK GENERATED DF <sub>1</sub> SMOKE/EXHAUST						M60A1 TANK ENGINE EXHAUST					
	15	60	120	180	240	300	15	60	120	180	240	300
	EXPOSURE TIME (MIN)						EXPOSURE TIME (MIN)					
SPRAGUE DAWLEY RAT	0/10	0/10	-	2/10	1/10	8/10	0/10	1/10	1/10	6/10	-	10/10
HARTLEY GUINEA PIG	0/10	0/10	7/10	8/10	-	10/10	0/10	0/10	-	1/10	0/10	10/10
TOTAL	0/20	0/20	7/10	10/20	1/10	18/20	0/20	1/20	1/10	7/20	0/10	20/20

TOTAL HYDROCARBON CONCENTRATION OF DF<sub>1</sub> SMOKE/  
EXHAUST

GRAB SAMPLES  
42.0 ± 16.8 MG/L  
(42,000 ± 16,800 MG/M<sup>3</sup>)

TOTAL HYDROCARBON CONCENTRATION OF ENGINE EXHAUST

ELECTROSTATIC PRECIPITATOR  
0.142 ± 0.10 MG/L  
(142 ± 100 MG/M<sup>3</sup>)

SPHEROCARB FILTER  
0.035 ± 0.039 MG/L  
(35 ± 39 MG/M<sup>3</sup>)

### BODY WEIGHT CHANGES

When survivor numbers were sufficient to statistically evaluate this parameter, no significant body weight loss was found among animals exposed for 15 and 60 minutes to DF2 and DF1 smoke and/or exhaust. At the 120-300 minute exposures, single incidences of weight loss were found among animals that had experienced extreme physiological stress (e.g. lethargy, hypopnea, nasal and oral frothiness, etc). Normal weight gain patterns, however, were noted in these animals by 14 days postexposure.

### HEMATOLOGY AND BLOOD CHEMISTRY

Evaluation of the hematology and blood chemistry data from animals used in these studies is incomplete. Preliminary examination of the data, however, suggests no significant effects on these parameters from the DF2 and/or DF1 smoke and/or exhaust exposures.

### RESPIRATORY MEASUREMENTS

Several Sprague-Dawley rats and Hartley guinea pigs may have shown changes in pulmonary resistance after 60 minute exposures to DF2 smoke and/or exhaust. Animals exposed to DF1 smoke and/or exhaust showed similar effects. Confirmation of these findings is awaiting completion of histopathologic evaluation of lung tissues.

## GROSS AND HISTOPATHOLOGY

### GROSS PATHOLOGY

Mottled lungs, accompanied with hemorrhage and edema were seen in Hartley guinea pigs and Fischer 344 rats exposed to DF2 smoke/exhaust for periods of 60 minutes or longer and in guinea pigs, B6C3F1 mice, Fischer and Sprague-Dawley rats exposed for the same time periods to DF2 exhaust.

Similar lung damage was found in guinea pigs exposed for 120 minutes or more to DF1 smoke/exhaust and Sprague-Dawley rats exposed for 60 minutes or longer to DF1 exhaust.

These effects were seen only in those animals that died spontaneously or within 24 hours after exposure to either type of fuel emission. Gross evidence of lung damage was negative in those animals that were sacrificed at 14 days postexposure.

### HISTOPATHOLOGY

To date, only histopathologic evaluation of organs from B6C3F1 mice and Hartley guinea pigs exposed to DF2 smoke and/or exhaust is complete. Findings with both species, however, indicate a direct relationship between reactive lung lesions and exposure of either sex to DF2 smoke and/or exhaust. In mice that died spontaneously from 300 minute exposures to DF2 smoke/exhaust, acute lung congestion in addition to acute multifocal bronchopneumonia was found. Animals that survived the exposure and were sacrificed 14 days later manifested diffuse lung congestion and subacute and chronic multifocal bronchopneumonia.

Exposure of mice to DF2 exhaust caused more severe pathology. Animals that died spontaneously from the 60 minute exposures showed diffuse lung congestion, pneumonia, lung necrosis and acute necrotizing tracheitis. Those that survived the 60-300 minute exposures and were sacrificed in 14 days showed chronic multifocal bronchopneumonia and in some cases multifocal lung congestion.

Histopathologic evaluation of organs from guinea pigs exposed for 60 minutes or more to DF2 smoke and/or exhaust also showed reactive lung lesions. Animals that died spontaneously showed acute diffuse interstitial pneumonia, acute diffuse bronchitis, acute diffuse lung hemorrhage, multifocal hemorrhage, and acute diffuse lung congestion. In addition, there was acute diffuse rhinitis with congestion in the turbinates and evidence of acute tracheitis. With those animals exposed 60-300 minutes and sacrificed in 14 days, acute and subacute multifocal bronchopneumonia, active chronic multifocal bronchopneumonia, and multifocal peribronchiolar lymphocytosis was found.

Guinea pigs exposed from 60-300 minutes to DF2 exhaust showed histopathologic respiratory lesions at spontaneous death and at 14 day sacrifice. Animals that died during or within 24 hours after exposure showed acute diffuse and/or multifocal congestion of the turbinates. Animals that survived the DF2 exhaust exposures and were sacrificed 14 days later manifested active chronic multifocal bronchiolitis and diffuse congestion of the turbinates.

In summary, it can be stated that 300 minute exposure to DF2 smoke/exhaust caused acute and chronic lung lesions in B6C3F1 mice. Similar effects were seen in guinea pigs exposed for 60-300 minutes. Exposure of both species to DF2 exhaust only caused acute and chronic lung damage by 60 minutes.

Both mice and guinea pigs failed to show significant lung lesions from 15 minute exposures to DF2 smoke and/or exhaust.

### DISCUSSION

These acute toxicity studies with M60A1 tank generated DF2 and DF1 smoke and/or exhaust represent unfinished work as not all the data have been evaluated. With the available information, however, several tentative remarks can be made about the significance of the findings.

First, the data imply that the toxic component of the clouds appears to be in the exhaust. This is evidenced by the fact that if a comparison is made between the concentrations of the DF2 or DF1 smoke/exhaust versus the concentrations of their respective exhausts, the total hydrocarbon content of the former is 163-237 times greater. Data show, however, that with exception of the guinea pig, which was more sensitive to the smoke/exhaust mixture, mortalities were similar with both types of clouds for both grades of fuel.

Based on this evidence, the question may then be asked: What common component caused the lethality in both types of clouds?

Available information from the literature indicates that it was not a depletion of oxygen or an excess of carbon monoxide or carbon dioxide. Normal atmospheric oxygen is 20.9%. In these studies with both DF2 and DF1 smoke and/or exhaust, the mean oxygen concentrations were 20% which is sufficient to prevent oxygen deficiency.

In regards to carbon monoxide and carbon dioxide levels, the mean concentrations never exceeded 80 or 8000 ppm, respectively. According to Patty (1963) these should not be lethal concentrations even at the exposure durations maintained in these studies.

The first comprehensive examination of the acute inhalation toxicity of diesel exhaust was conducted by Pattle et al. (1957). Their objective was to determine the principal toxic constituents of diesel exhaust generated under various "load" conditions of engine operation. Mice, rabbits, and guinea pigs were exposed for 7-14 hours to the undiluted diesel exhaust under light load conditions. The exposures produced nearly complete mortality in all species accompanied by mild pathologic alterations in the trachea and lung. Aldehydes (16 ppm) and oxides of nitrogen (46 ppm) were presumed to be the primary toxic agents in this case.

The effects these components had on the mortalities in the DF2 and DF1 smoke and/or exhaust studies require further exploration. Similar measurements were made but require substantiation with other instrumentation. One comment can be made, however, with regards to the concentrations of nitrogen dioxide in DF2 and DF1 smoke and/or exhaust clouds. Several NO<sub>2</sub> measurements were taken (Table 6) from both type clouds generated from both fuels during the

course of these tests. The maximal reading recorded was found with DF1 smoke/exhaust and equaled 40 ppm. This concentration should not be lethal in the species tested according to Latowsky et al. (1941). They showed that  $\text{NO}_2$  concentrations greater than 100 ppm are required to cause death in cats, guinea pigs, mice, rats, and rabbits.

In addition, the concentration of sulfur dioxide and ethylene oxide never exceeded 20 ppm or 0.1 ppm, respectively, in any of the tests. These concentrations should not be lethal. Weedon et al. (1939) exposed mice and guinea pigs to concentrations of 10, 25, 33, 65, 100, 150, 300, and 1000 ppm of sulfur dioxide and found that the gas caused no significant effects in either species at concentrations of 33 ppm or less. They also determine the median lethal concentration for mice which was found to be 130 ppm for 24 hours, 340 for 6 hours, 610 for 1 hour and 1350 ppm for 10 minutes.

Ethylene oxide concentrations (e.g. < 0.1 ppm) found in these studies with DF2 and DF1 smoke and/or exhaust were also found to be non-lethal. Patty (1963) reports that concentrations of 560-600 ppm for 8 hours are required to cause death in guinea pigs and 1100 ppm for the same exposure period in rats.

One more facet should be addressed with regards to cause of lethality in these studies. That is, the hydrocarbon particulate sizes of the smoke and/or exhaust clouds. Schreck (1978) reported that one reason why increased exposure to particulates derived from diesel engine exhaust may constitute a potential health hazard is because of size. His work showed that the carbonaceous particles are primarily in a size range (0.2 - 0.3  $\mu\text{m}$  mass median diameter) which could allow for deposition and possible retention in the deep lung compartments. Measurements of particulate sizes from the described DF2 and DF1 smoke and/or exhaust clouds showed that at least 98% of the particles had mean diameters of less than 0.1 micron. Based on Schreck's hypothesis, this would make them prime candidates for deposition and retention in the lower lung compartments.

These studies do not address the effects of repeated exposures nor the possible effects on reproductive processes, including long term carcinogenic potential. Some of these factors are being addressed in a 13 week daily exposure test study of laboratory animals to lower concentrations of DF2 smoke and/or exhaust now in progress.

### CONCLUSIONS

M60A1 tank generated DF2 smoke and/or exhaust disseminated under static airflow conditions caused death in laboratory animals after 60-300 minute exposures to mean total hydrocarbon concentrations of 45,600  $\text{mg}/\text{M}^3$  of smoke/exhaust and 280  $\text{mg}/\text{M}^3$  of exhaust. DF1 smoke and/or exhaust caused similar effects from 120-300 minute exposures to mean concentrations of 42,000  $\text{mg}/\text{M}^3$  of smoke/exhaust and 60-300 minute exposures to 177  $\text{mg}/\text{M}^3$  of exhaust.

The significant chemical component causing the toxicity is postulated to originate from DF2 and DF1 exhaust.

It is postulated that death results from toxic and/or pathologic effects on the respiratory system.

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## COMPARATIVE STUDIES OF THREE GUINEA PIG SENSITIZATION TESTS

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### INTRODUCTION

A modified form of the Landsteiner-Draize Guinea Pig Sensitization method has been used exclusively at the Toxic Hazards Research Unit for sensitization testing. Critics of the Landsteiner test contend the method fails to detect many known weak to moderate human sensitizers. In the past few years many additional sensitization methods have been developed. Comparison studies of various methods with the Landsteiner test are reported in the literature, but none have been conducted at this laboratory. It was decided that it would be of interest to conduct our own comparison of the Landsteiner test with some of the more well established alternate methods. The Guinea Pig Maximization Test, G.P.M.T., (Magnusson and Kligman, 1969) and the Maguire Test (Maguire, 1973) were selected. The Maguire Test, the G.P.M.T., and the Landsteiner represent three different modes of administration in their respective induction phases. The Landsteiner Test uses intradermal injections, the Maguire Test uses topical applications and the G.P.M.T. uses both injections and topical applications. The Maguire Test was recommended by and used at Dow Laboratories, (private communication, V. K. Rowe, 1978). The G.P.M.T. is probably the next most well established method after the Landsteiner test. Also, the G.P.M.T. and Maguire Test are relatively simple to perform and require no special equipment.

Three materials of known sensitization potential were selected. Dinitrochlorobenzene (DNCB) was selected as a severe sensitizer (Klecak et al., 1977). Carbowax 4000 was selected as the nonsensitizer. Formaldehyde was chosen as a known weak to moderate sensitizer (Magnusson and Kligman, 1969). After visual evaluation of the animals, skin sections were taken from the application sites of all animals of the studies for histologic evaluation. Evidence exists that sensitizing agents can be quantitatively identified by measuring the degree of mononuclear cell infiltration in the dermis of exposed animals (Groth, 1978).

### MATERIALS AND METHODS

Dinitrochlorobenzene (practical grade), formaldehyde (37% aqueous), and Carbowax PEG 4000 (polyethylene glycol 4000) powder (laboratory grade) were obtained from Fisher Scientific Company.



The concentrations used were based on the results of the preliminary primary initiation tests. Second trials with formaldehyde were conducted to determine the effect of concentration.

Hartley strain, female albino guinea pigs 6 to 8 weeks of age obtained from Murphy Breeding Laboratory, Plainfield, Indiana were utilized for all studies. Twenty animals were used per group.

Skin sections were taken after the 48 hour evaluation for microscopic observation.

## METHODS

### LANDSTEINER

On test day 1, the guinea pigs were weighed and clipped on the scapular areas. 0.05 ml of 0.1% dilutions of the test materials were injected intradermally into the upper right scapular area of each animal. Similar injections of the vehicle were made into the left scapular area. The injection sites were evaluated and scored numerically at 24 and 48 hours post injection. On test days 3, 5, 8, 10, 12, 15 and 17, 0.1 dilutions of the test materials were made into the clipped dorsal lumbo-sacral area of each animal. After a 3 week incubation period the animals were challenged with 0.05 ml injections of the test materials and respective vehicles in the right and left scapular areas. These injection sites were evaluated and scored at 24 and 48 hours post injection. The final sensitization score for each animal was calculated with the following formula: (day 1 test material score - day 1 vehicle score) - (challenge, test material score - challenge vehicle score).

### MAGUIRE TEST

On day 1 an area on the back of each animal directly above the forelegs was clipped and chemically depilicated. Solutions of 0.1 ml of non-irritating concentrations of the test materials were applied to these areas on  $\frac{1}{2} \times \frac{1}{2}$  inch squares of cotton gauze. These patches were covered with dental dam then tightly occluded with elastic adhesive bandages. On day 3 the patches were removed and a second patch was applied to the same site and again occluded. On day 5 the patches were removed and a total volume of 0.2 ml Freund's Adjuvant was injected in 2 points adjacent to the insult site in each animal. Patches containing 0.1 ml of the test material were again applied. On day 8 induction patches were again applied and occluded. The patches were removed 48 hours later and the animals were rested for 2 weeks. After the 2 week incubation period both flanks of the animals were clipped and the animals were challenged with 0.1 ml applications of the test materials on one flank and the skin responses at the challenge sites were recorded after 24 and 48 hours. Any animal that demonstrated erythema or edema was rated as a positive responder.

### GUINEA PIG MAXIMIZATION TEST

The guinea pig maximization test, G.P.M.T., utilized separate groups of animals for evaluation of the test materials and their vehicles. On day 1 the shoulder regions of the test material animals were clipped as closely as possible.

Three pairs of intradermal injections were made so that on each side of the midline of the back there was 1 row of 3 injection sites. The injection sites were within a 2 x 4 cm area and consisted of: 1) 0.1 ml of Freund's Adjuvant (F.C.A.); 2) 0.1 ml test materials, and; 3) 0.1 ml test material in F.C.A. The control group received the following pairs of intradermal injections: 1) 0.1 ml F.C.A.; 2) 0.1 ml vehicle (solvent), and; 3) 0.1 ml vehicle plus F.C.A. On day 7 the shoulder area was again clipped, then shaved with a safety razor. 12 x 4 cm filter paper patches saturated with the test material or vehicle and were applied over the injection sites. Dental dam was placed over the patch and tightly secured with an elastic adhesive bandage. On day 21 both flanks of all animals were shaved and saturated patches were occluded. The left side patch was saturated with the test materials and the right side patch with the appropriate vehicles. The challenge sites were evaluated at 24 and 48 hours post injection. Guinea pigs of the test material group that demonstrated greater responses to the test materials than the control group were rated as positive.

## RESULTS

### THE LANDSTEINER TEST

Table 1 summarizes the results of the gross evaluation of the Landsteiner tests. The 24 and 48 hour mean reaction scores for dinitrochlorobenzene are extremely high and indicate a strong sensitization reaction. The first trial with formaldehyde produced a very mild sensitization response as indicated by low mean reaction scores in a low percentage of the animals at 24 hours. At 48 hours post challenge, no sensitization was evident. In the second trial a very mild sensitization reaction was noted. However, the reaction was found only at the 48 hour examination. The reactions to formaldehyde are questionable in that there was a similar reaction elicited by Carbowax, a known human non-sensitizer. There were histologic changes evident in both the peanut oil vehicle and dinitrochlorobenzene injection sites. There were increased incidences of eosinophilia and scab formation in formaldehyde injection sites. Lesions attributable to the administration of Carbowax 4000 were not noted.

TABLE 1. LANDSTEINER GROSS EVALUATION

	Dinitrochloro- benzene	Test Materials		
		Carbowax 4000	Formaldehyde	
			Test #1	Test #2
Conc. (%)	0.1	0.1	0.1	0.1
Vehicle	Peanut Oil	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O
$\bar{x}$ Reaction Scores				
at 24 hrs.	653	44	51	0
at 48 hrs.	403	50*	0	40
% Positive Animals				
at 24 hrs.	90	20	15	0
at 48 hrs.	75	5	0	32

\* Only 1 Animal.

## THE MAGUIRE TEST

Dinitrochlorobenzene and formaldehyde were obvious sensitization agents in the Maguire Test. Table 2 shows that at 24 hours 76 and 89% of the animals demonstrated gross positive sensitization response to dinitrochlorobenzene and formaldehyde, trial 1, respectively. At 48 hours, 71 and 84% of the animals were still positive. A response of 65% or greater is considered indicative of a strong sensitizer in the Maguire Test. The severity of mononuclear inflammatory reaction was increased in the dinitrochlorobenzene and formaldehyde application sites. The frequencies of acantholysis and scab formation were increased as a result of dinitrochlorobenzene or formaldehyde applications. Formaldehyde also caused increases in acute inflammation, spongiosis and eosinophilic infiltrations.

A second trial with formaldehyde was conducted using a lower concentration. At the lower formaldehyde concentration, the percentage of positive responders was considerably reduced (Table 2). No tissues were taken from the second trial.

There was no indication of a sensitization response with Carbowax 4000.

TABLE 2. MAGUIRE TEST GROSS EVALUATION

	<u>Test Materials</u>			
	<u>Dinitrochloro- benzene</u>	<u>Carbowax 4000</u>	<u>Formaldehyde</u>	
			<u>Test #1</u>	<u>Test #2</u>
Conc. (%)	0.1	10	37-10*	5
Vehicle	Acetone	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O
% Positive Animals				
at 24 hrs.	76	0	89	21
at 48 hrs.	71	0	84	5

\* 37% used on first application, 10% used thereafter.

## THE GUINEA PIG MAXIMIZATION TEST

The results of the gross evaluation of the G.P.M.T. are summarized in Table 3. Dinitrochlorobenzene was found to be a strong sensitizer. At 24 and 48 hours, 95 and 100% of the animals had positive sensitization responses, respectively. Dinitrochlorobenzene administration resulted in increased severity and frequency of mononuclear inflammatory reactions, and increased frequencies of acute inflammatory reactions, spongiosis, acantholysis, eosinophilic infiltration and scab formation.

Formaldehyde, trial 1, demonstrated no sensitization potential when evaluated grossly. The microscopic evaluation revealed increases in the frequencies of acute inflammatory reactions and eosinophilic infiltration.

TABLE 3. GUINEA PIG MAXIMIZATION TEST  
(G.P.M.T.) GROSS EVALUATION

	<u>Test Materials</u>			
	Dinitrochloro-	Carbowax	<u>Formaldehyde</u>	
	<u>benzene</u>	<u>4000</u>	<u>Test #1</u>	<u>Test #2*</u>
Conc. Inj. (%)	0.1	5	0.1	0.2
Conc. Top. (%)	0.1	10	5	5
Vehicle	Peanut Oil	H <sub>2</sub> O	H <sub>2</sub> O	H <sub>2</sub> O
% Positive Animals				
at 24 hrs.	95	0	0	25
at 48 hrs.	100	0	0	25

\* Sodium lauryl sulfate was applied to the animals 24 hrs. prior to the topical application.

A second G.P.M.T. trial was conducted using higher concentrations of formaldehyde. Also, a 10% sodium lauryl sulfate solution was applied 24 hours prior to the topical induction patch in the second trial to induce mild irritation in the test area. Gross evaluation of the second trial with formaldehyde demonstrated a slight sensitization response as 25% of the animals had positive readings at 24 hours post challenge. No tissues were evaluated in the second trial.

Grossly, Carbowax 4000 demonstrated no sensitization reaction; however, an increase in the frequency of spongiosis was observed.

#### SUMMARY

The known sensitization potential of dinitrochlorobenzene was confirmed by the Landsteiner Test. The mean reaction scores of formaldehyde (Trials 1 and 2) and Carbowax 4000 were quite low and essentially equal. If these are interpreted as negligible responses, then the known sensitization potential of formaldehyde was not confirmed. However, if the scores are interpreted as positive sensitization responses, the known non-sensitizing capacity of Carbowax 4000 was not confirmed, leading to a false positive conclusion.

The known sensitization potential of all of the test materials was confirmed by the Maguire Test. The difference in the percentage of positive responders in the formaldehyde trials indicate that the method is concentration dependent.

Dinitrochlorobenzene was confirmed as a strong sensitizer while Carbowax 4000 was a non-sensitizer by the G.P.M.T. None of the animals demonstrated a positive response to formaldehyde in the first trial. Increasing the concentration of the injections and inducing primary irritation prior to the application of the topical induction patch in the second trial appeared to increase the sensitivity of the test, as 25% of the animals responded. This would indicate formaldehyde to be a sensitizer.

Groth (1978) has suggested that the degree of mononuclear cell infiltration is an indication of a sensitization reaction. Increases in the mononuclear inflammatory reactions with formaldehyde and D.N.C.B. (known sensitizers) were noted in both the Maguire Test and the G.P.M.T. These parameters were unchanged in the Carbowax 4000 groups.

The Maguire Test and G.P.M.T. appeared to be the most reliable indicators of the sensitization potential of the three materials, provided adequate concentrations are used.

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## URINARY METABOLITES OF INHALED METHYLCYCLOHEXANE

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### INTRODUCTION

The components of urine have been studied for many years. There are numerous chromatographic methods for isolating portions for study. Most methods are specific and lengthy. Liquid chromatographic methods are more generally suitable for separation of the components in urine (Burtis and Warren, 1968; Fiala et al., 1976; Jolley et al., 1970; Scott et al., 1970; Young, 1970). For volatiles, however, gas chromatography is generally faster, more sensitive and interfaces easily to a mass spectrometer for identification (Dowty et al., 1976; Finkle et al., 1971; Horning and Horning, 1971; Lewis et al., 1979; Teranish et al., 1972; Zlatkis and Liebich, 1971; Zlatkis et al., 1973; Zlatkis et al., 1979).

Capillary gas chromatography was used in this study to separate urinary volatiles. The volatiles present in the urine from methylcyclohexane exposed rats but not present in control rat urine were identified by mass spectrometer and confirmed by GC retention time.

### EXPERIMENTAL

The rats used in this study were part of a group being exposed for one year to 400 ppm methylcyclohexane on a 6 hour/day, 5 day/week schedule. Urine samples were collected overnight from Fischer 344 control and exposed rats from Charles River Breeding Laboratories held in Hoeltge metabolic cages. During collection the rats had free access to water and food. The food they were accustomed to eating was powdered to prevent it from being carried into the collection cage. The collected urine was frozen (approximately -10°C) until testing.

Initial capillary GC investigations demonstrated that a 50 m x 0.25 mm Carbowax 20M column was satisfactory for the separation of urine volatiles yielding very sharp, well-separated peaks. The procedure yielding the most reproducible results was direct injection of 5 microliters of urine into an injector sleeve filled with glass beads to retain the non-volatile urine components. The splitter was set to allow 10% of the injected volatiles into the column. This method did not lead to plugging and provided peaks of reasonable intensity. Figure 1 illustrates

the chromatograms obtained from control and exposed rat urine. The most significant difference between the control and exposed rats is the appearance of 12 peaks, most of which are in the range from 38.5 to 41 minutes. Fortunately, these peaks appear in an area which is relatively free of components in control urine.

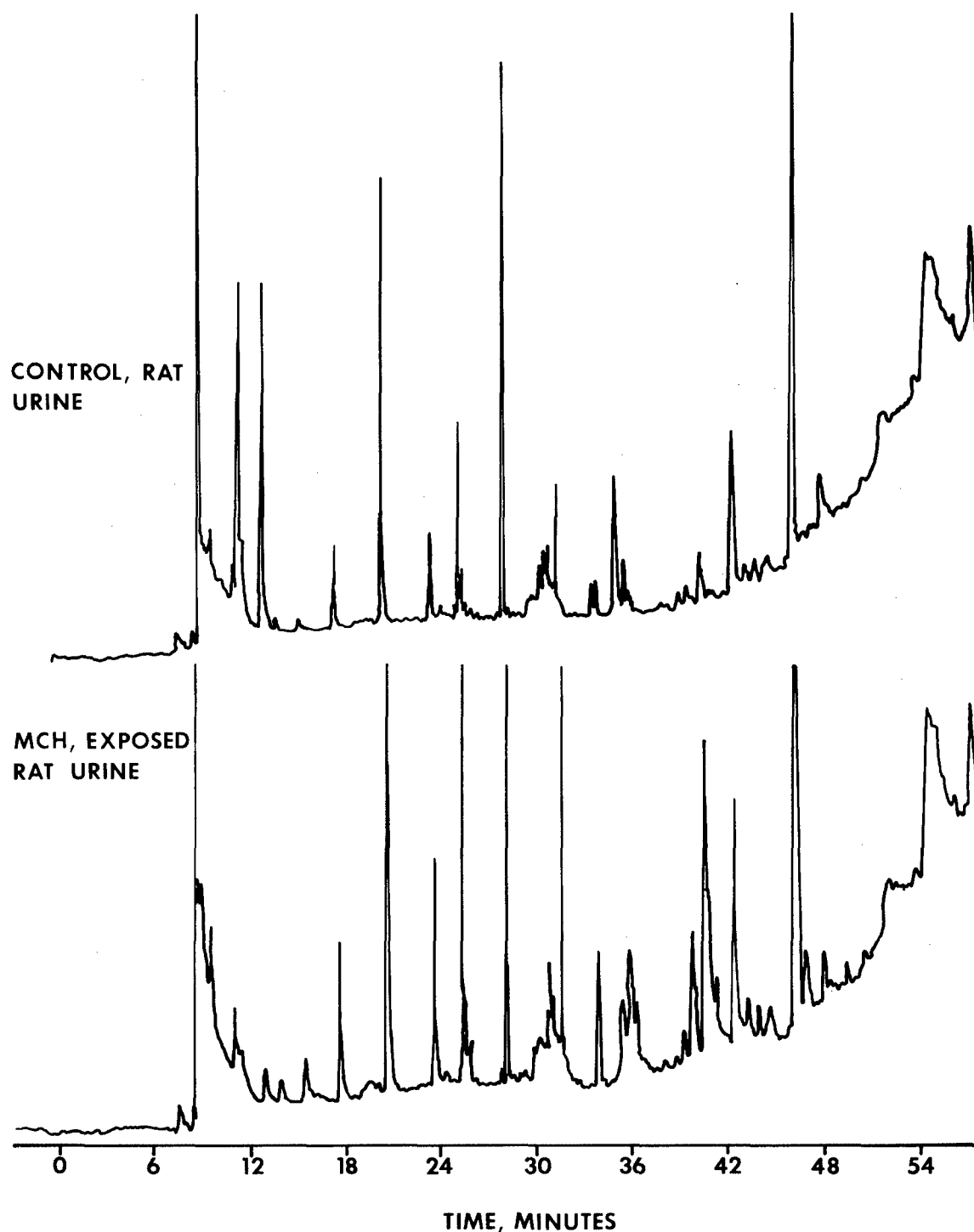


Figure 1.

Once it had been shown that new peaks, presumably metabolites, had appeared in the GC of exposed rat urine, the analytical methodology was applied to GC/MS analysis under the following conditions:

Hewlett-Packard 5993 GC/MS  
Injection Size = 5  $\mu$ l  
Injector sleeve cleaning after each injection  
Water injection check after each cleaning  
Initial Oven Temperature = 32°C  
Temperature Rate = 4°C/min  
50 m x 0.25 mm Carbowax 20M capillary column  
20 to 320 amu MS scan

Again, a group of peaks, not present in control urine, appeared in the total ionization GC of exposed rat urine with retention times of 17 to 22 minutes as shown in Figure 2.

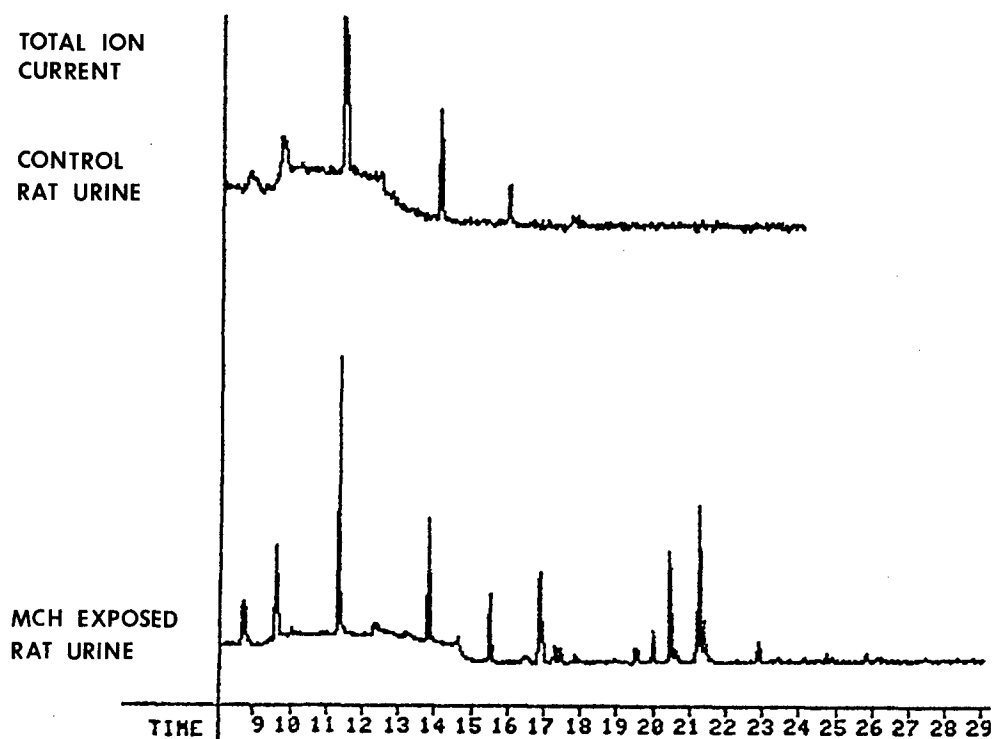


Figure 2.

Mass spectral comparison of these peaks with the National Bureau of Standards and Wiley Libraries by Hewlett-Packard's Probability Based Searching method, identified these peaks as methylcyclohexanols. Known additions of cis and trans 2-, 3-, and 4-methylcyclohexanols to exposed rat urine were made to check the retention times (Figure 3). The 1-methylcyclohexanol was received too late for



known addition but the retention time matched for the same chromatographic conditions (Figure 4).

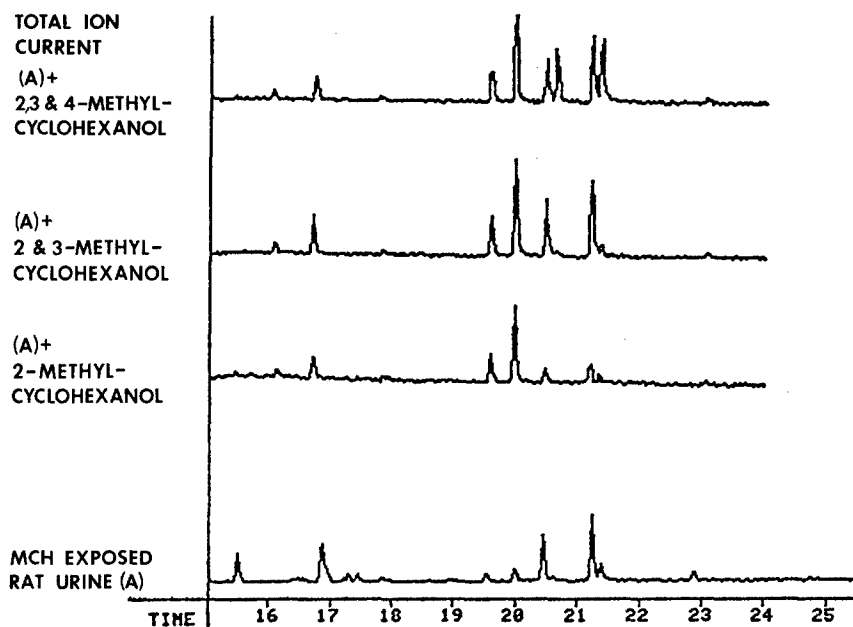


Figure 3.

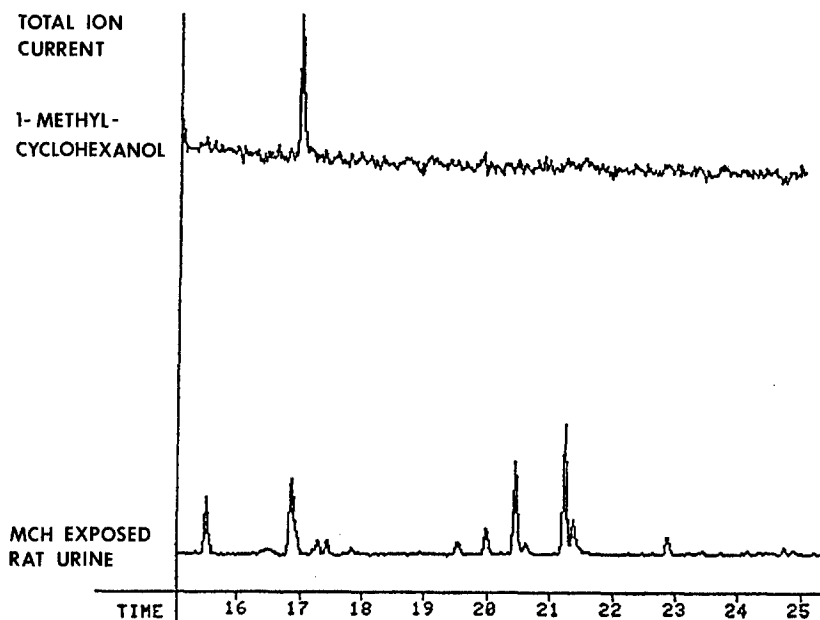


Figure 4.

Comparison of the mass spectra of trans 2-methylcyclohexanol with the metabolite of identical retention time is shown in Figure 5. Similar matches were obtained for the other peaks with their respective knowns.

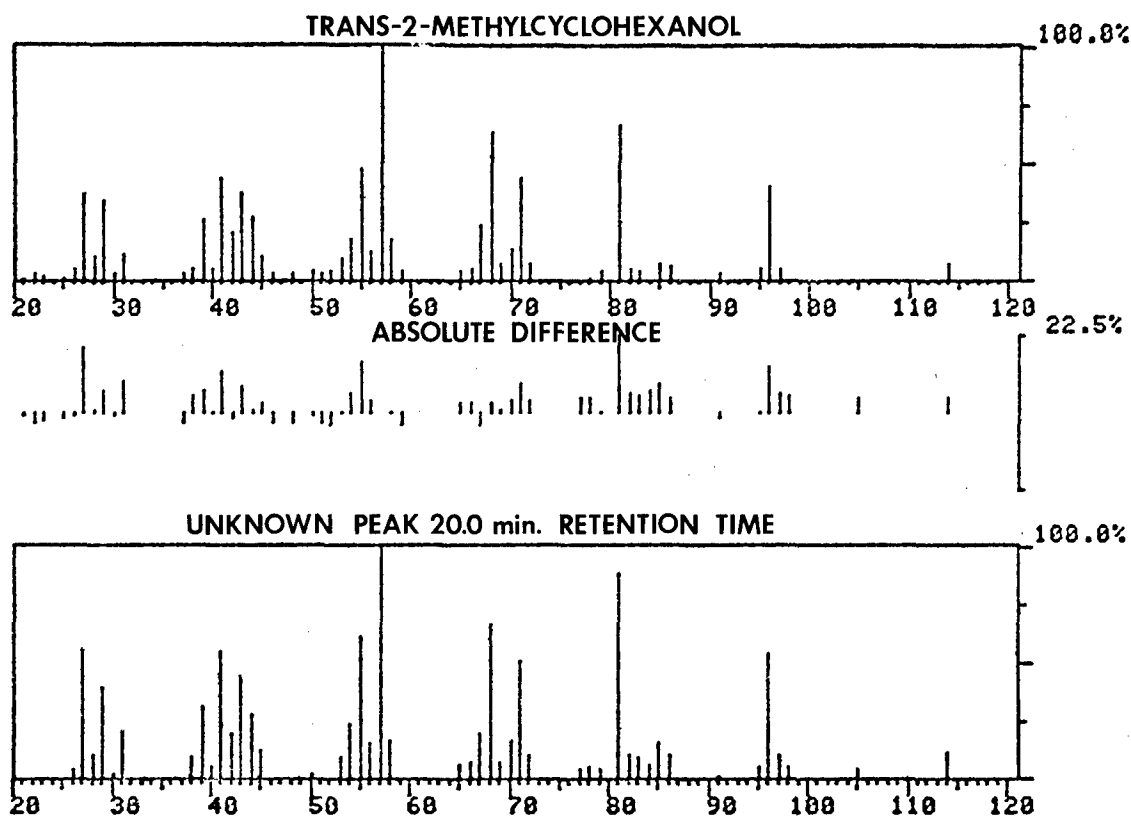


Figure 5.

On the basis of GC/MS analysis, all positional and geometric isomers of methylcyclohexanol have been identified in the urine of rats exposed to 400 ppm methylcyclohexane. No evidence of the presence of any methylcyclohexanone was obtained.

## RESULTS

The separation method for urinary volatiles was found to be fast and comparatively simple. The capillary column gave a good separation of the many volatiles in the urine. Methylcyclohexane inhaled by rats was metabolized to 1-methylcyclohexanol and cis and trans 2-, 3- and 4-methylcyclohexanols. Further oxidation to methylcyclohexanones was not evident. The trans 3-methylcyclohexanol was the isomer of the greatest quantity as determined by peak area on the mass chromatogram. It was 30.1% of the total methylcyclohexanol area (Table 1).

TABLE 1.

<u>COMPOUND</u>	<u>RETENTION TIME</u>	<u>AREA %</u>
1-methylcyclohexanol	16.9 min	25.9
cis 2-methylcyclohexanol	19.5 min	4.3
trans 2-methylcyclohexanol	20.0 min	6.3
cis 3-methylcyclohexanol	20.4 min	21.7
cis 4-methylcyclohexanol	20.6 min	2.5
trans 3-methylcyclohexanol	21.2 min	30.1
trans 4-methylcyclohexanol	21.4 min	9.2
		100.0

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## AQUATIC TOXICITY OF FUEL SYSTEM ICING INHIBITOR

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### INTRODUCTION

Jet aircraft fuels are often augmented with compounds to prevent ice formation, and to inhibit growth of microorganisms in fuel storage tanks (Thomas and Hill, 1977; Neihef and Bailey, 1978). One compound often used is ethylene glycol monomethyl ether (EGME). The Army Environmental Hygiene Agency (EHA), as environmental consultant to the Defense Logistics Agency (DLA), was asked to evaluate the aquatic toxicology potential of this material. The USAF Occupational and Environmental Health Laboratory (USAF OEHL) was requested by the EHA to provide additional support for this study. Dawson et al. (1975) reported 96-hour  $LC_{50}$ 's of EGME to be greater than 10,000 ppm for both blue gill and tidewater silverside fish.

As there were relatively little other data available in the literature on EGME, especially on freshwater species, laboratory bioassays were performed by both organizations. The primary tests were static bioassays performed with crayfish (*Procambarus* spp.), the fathead minnow (*Pimephales promelas*), and a mosquito larva (*Wyeomyia smithii*). Both static and dynamic bioassays were performed on the fathead minnow, the most commonly used test organism of the aquatic toxicology world. Additionally, confirmation testing was done on several other organisms including catfish (*Ictalurus* spp.), rainbow trout (*Salmo gairdneri*), killifish (*Fundulus* spp.), and mosquito fish (*Gambusia affinis*).

### MATERIALS AND METHODS

Both the static and dynamic aquatic toxicity tests were standard, 96-hour  $LC_{50}$  studies, performed according to the procedures outlined in Standard Methods (1975). The test material was pure, analytical grade EGME, obtained from DLA. Water temperature, pH, and dissolved oxygen levels were monitored throughout each of the test periods.

The acute toxicity for EGME for each different test organism was calculated using a Statistical Analysis package program for probit analysis (Barr et al., 1979) run on an IBM Series 600 computer.

The results are expressed as the 96-hour  $LC_{50}$ , the concentration of EGME that was lethal to 50% of the test organisms.

## RESULTS AND DISCUSSION

The acute toxicities calculated from the static bioassays performed on the three main test organisms were: fathead minnow, 2,380 ppm; crayfish, 2,600 ppm; mosquito larvae, 29,200 ppm. The dynamic bioassay for the fathead minnow resulted in an acute toxicity of 1,400 ppm (Figures 1-4). Static bioassays were also performed using mosquito fish (*Gambusia affinis*), catfish (*Ictalurus sp.*), killifish (*Fundulus sp.*), and rainbow trout (*Salmo gairdneri*). The acute toxicity thresholds (lowest tested concentration where death did not occur) for the fish were 10,000 ppm, 7,500 ppm, 2,500 ppm and 10,000 ppm, respectively.

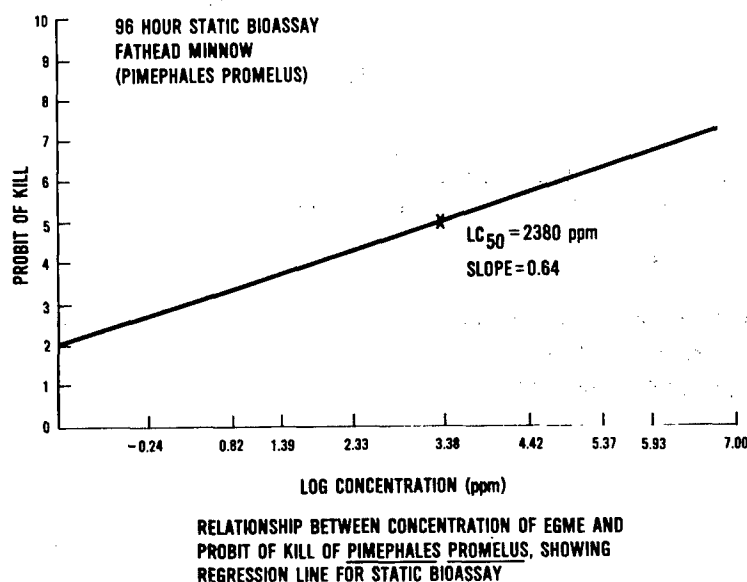


Figure 1.

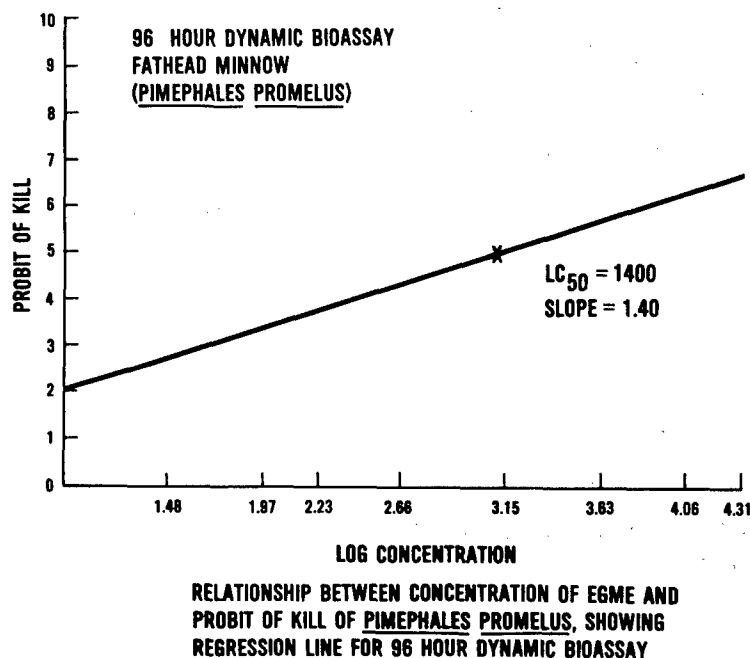


Figure 2.

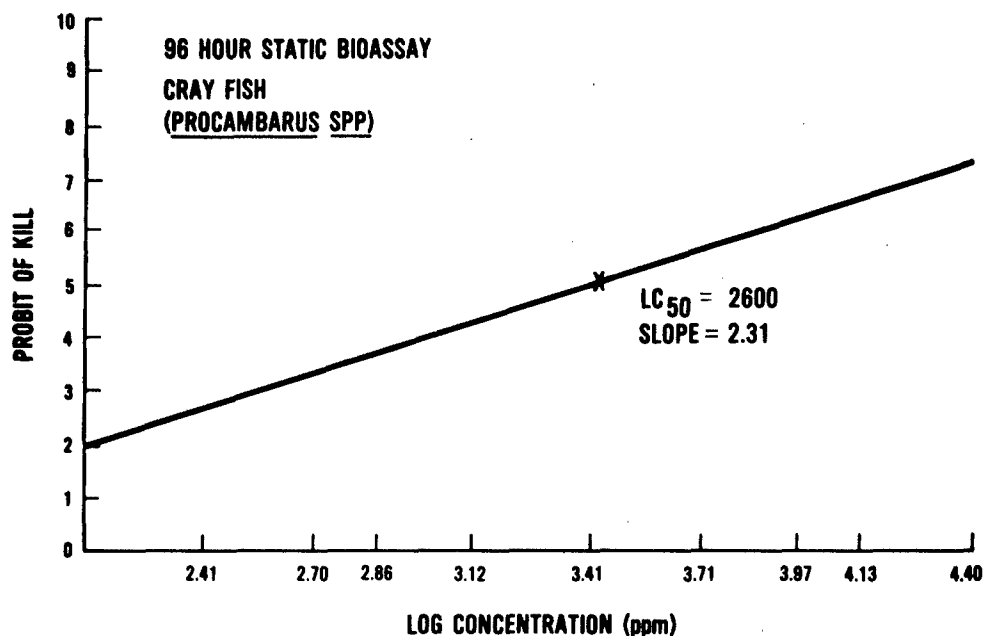


Figure 3.

RELATIONSHIP BETWEEN CONCENTRATION OF EGME AND  
PROBIT OF KILL OF PROCAMBARUS SPP, SHOWING  
REGRESSION LINE FOR 96 HOUR STATIC BIOASSAY

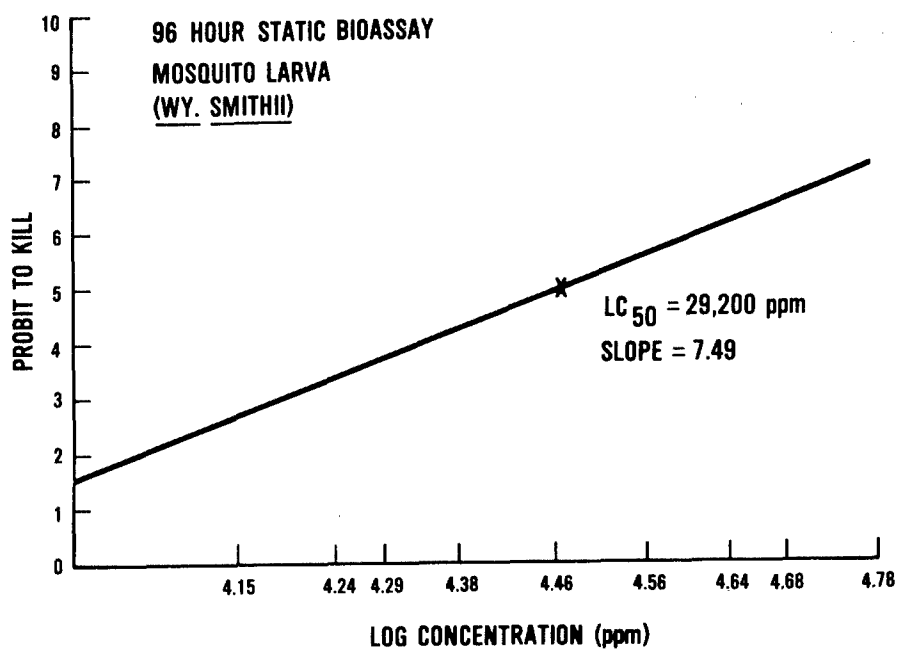


Figure 4.

RELATIONSHIP BETWEEN CONCENTRATION OF EGME AND PROBIT OF KILL  
OF WY. SMITHII LARVA, SHOWING REGRESSION LINE FOR 96 HOUR STATIC  
BIOASSAY CONFIDENCE INTERVAL (95%) FOR  $LC_{50}$  IS 23,100 TO 34,700 ppm

Applying a safety factor of 0.1 to the 1,400 ppm concentration that resulted in 50% mortality in the dynamic bioassay for the fathead minnow (the most sensitive test organism) would result in a safe level for the aquatic environment of 140 ppm. The toxicity of JP-4 jet fuel is approximately 5 ppm and the concentration of EGME in the fuel is usually in the range of 0.1 to 0.15%; therefore in normal USAF usage, the EGME would not be an acute aquatic toxicant in itself as the greater toxicity of the JP-4 would have a masking effect on the toxicity of the EGME component.

In any environmental incident where EGME is discharged into the aquatic environment without dilution in JP-4, it should not have any effect unless the concentration is greater than 140 ppm.

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BIOCHEMICAL CHARACTERIZATION OF KIDNEY  
LESIONS FROM HYDROCARBON FUELS

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INTRODUCTION

Male rats were exposed to JP-5 fuel vapor in an intermittent fashion for 6 hours a day, 5 days a week. Exposures were for 30 or 80 days at vapor concentration levels of 500 and 900 mg/m<sup>3</sup>. One additional series of exposures was performed using decane at 7500 mg/m<sup>3</sup> for 25 days.

After completion of the exposure, the male rats were placed in metabolic chambers and urine was collected. The urine was assayed for urea, creatinine and gamma-glutamyl transpeptidase (GGT). The animals were killed and tissue samples were taken for histologic examination. Blood samples were taken. The blood samples were assayed for erythrocyte count, hemoglobin, serum alanine transaminase (ALT), serum aspartate transaminase (AST), serum GGT, serum creatinine, and blood urea nitrogen (BUN). From these values, creatinine clearance (ml/min/kg), urea clearance (ml/min/kg), and GGT excretion (IU/mg creat) were calculated (30 day exposure only).

In all the 80 day exposure studies, the serum creatinine levels were elevated and in two of these studies the creatinine clearances were decreased. In only one of these studies the BUN was significantly elevated and all the urea clearance changes appeared to be unrelated to exposure to the fuels. None of these measurements was significantly changed in the 30 day exposures. However, there was a significant increase in the urinary activity of GGT per mg of creatinine excreted in the animals exposed to shale JP-5 for 30 days at 900 mg/m<sup>3</sup>. The tissue examinations have been completed for two of the 80 day exposures and no renal lesions were found as a result of hydrocarbon exposure.

These findings are in agreement with those of Gaworski, MacEwen, and Vernot where they exposed rats to fuel vapor of petroleum JP-5 and DFM and shale JP-5 and DFM. Their exposures were for 90 days (24 hrs/day) at concentrations up to 750 mg/m<sup>3</sup>. Besides the similar clinical changes, they also described a histopathologic renal lesion in male rats.

Male rats that are exposed to hydrocarbon fuels are developing a renal lesion which is disturbing renal function (serum creatinine and creatinine clearance), but only after a long exposure (>80 days) to the fuel vapor. The urinary activities of the brush-border enzyme-GGT show some promise of early detection of this renal lesion.

### OBJECTIVE

To measure changes in the standard renal function studies of male rats after exposure to hydrocarbon fuels.

### MATERIALS

Petroleum JP-5	-	Standard MILSPEC JP-5
Shale JP-5	-	JP-5 produced from a 100,000 barrel refinement of Colorado oil shale
Decane	-	Eastman Kodak technical grade
Rats	-	Male Fischer 344
Assay Kits	-	Worthington Diagnostics

### CLINICAL TEST MEASUREMENTS

#### SERUM ASSAYS

ALT (SGPT)

AST (SGOT)

Creatinine

Blood Urea Nitrogen  
(BUN)

#### URINE ASSAYS

Urea

Creatinine

Total Volume

Gamma Glutamyl Trans-  
peptidase (GGT)\*

\* Only performed for studies C, D, E.

TABLE 1. CHANGES IN SERUM CREATININE VALUES OF MALE RATS AFTER EXPOSURE TO HYDROCARBON VAPOR

HYDROCARBONS	GROUP N	EXPOSURE DAYS	VAPOR CONCENTRATION (mg/m <sup>3</sup> )	SERUM CREATININE (mg/dl)	
				CONTROL	EXPOSED
A. Pet. JP-5	18	80	500	0.67 (0.03) <sup>†</sup>	0.72 (0.02)*
B. Shale JP-5	18	80	500	0.67 (0.03)	0.87 (0.03)*
C. Shale JP-5	18	80	500	0.69 (0.03)	0.83 (0.04)*
D. Shale JP-5	6	30	900	0.65 (0.06)	0.67 (0.11)
E. Decane	6	28	7500	0.62 (0.08)	0.70 (0.05)

<sup>†</sup> Mean (standard error)

\* Significantly different from control values (p < 0.05)

TABLE 2. CHANGES IN CREATININE CLEARANCE VALUES OF MALE RATS AFTER EXPOSURE TO HYDROCARBON VAPOR

HYDROCARBONS	GROUP N	EXPOSURE DAYS	VAPOR CONCENTRATION (mg/m <sup>3</sup> )	CREATININE CLEARANCE (ml/min/kg)	
				CONTROL	EXPOSED
A. Pet. JP-5	18	80	500	1.78 (0.16) <sup>†</sup>	2.47 (0.29)
B. Shale JP-5	18	80	500	2.33 (0.18)	2.03 (0.13)
C. Shale JP-5	18	80	500	3.46 (0.18)	2.75 (0.15)*
D. Shale JP-5	6	30	900	3.91 (0.18)	3.76 (0.22)
E. Decane	6	28	7500	4.57 (0.90)	3.54 (0.21)

<sup>†</sup> Mean (standard error)

\* Significantly different from control values (p < 0.05)

TABLE 3. CHANGES IN BUN LEVELS OF MALE RATS AFTER EXPOSURE TO HYDROCARBON VAPOR

HYDROCARBONS	GROUP N	EXPOSURE DAYS	VAPOR CONCENTRATION (mg/m <sup>3</sup> )	BUN (mg/dl)	
				CONTROL	EXPOSED
A. Pet. JP-5	18	80	500	15.3 (0.4) <sup>†</sup>	17.9 (0.7)*
B. Shale JP-5	18	80	500	15.6 (0.3)	16.0 (0.5)
C. Shale JP-5	18	80	500	20.4 (0.9)	16.5 (0.5)*
D. Shale JP-5	6	30	900	20.3 (1.8)	20.7 (2.2)
E. Decane	6	28	7500	18.4 (0.8)	18.3 (1.1)

<sup>†</sup> Mean (standard error)

\* Significantly different from control values (p < 0.05)

TABLE 4. CHANGES IN UREA CLEARANCE VALUES OF MALE RATS AFTER EXPOSURE TO HYDROCARBON VAPOR

HYDROCARBONS	GROUP N	EXPOSURE DAYS	VAPOR CONCENTRATION (mg/m <sup>3</sup> )	UREA CLEARANCE (ml/min/kg)	
				CONTROL	EXPOSED
A. Pet. JP-5	18	80	500	1.17 (0.11) <sup>†</sup>	1.65 (0.18)
B. Shale JP-5	18	80	500	2.21 (0.16)	1.62 (0.08)*
C. Shale JP-5	18	80	500	2.38 (0.14)	2.68 (0.16)
D. Shale JP-5	6	30	900	2.66 (0.18)	2.29 (0.22)*
E. Decane	6	28	7500	2.33 (0.15)	2.38 (0.22)

<sup>†</sup> Mean (standard error)

\* Significantly different from control values (p < 0.05)

TABLE 5. CHANGES IN TOTAL VOLUME OF URINE OF MALE RATS AFTER EXPOSURE TO HYDROCARBON VAPOR

HYDROCARBONS	GROUP N	EXPOSURE DAYS	VAPOR CONCENTRATION (mg/m <sup>3</sup> )	TOTAL VOLUME (ml/24 hrs)	
				CONTROL	EXPOSED
C. Shale JP-5	18	80	500	12.2 (0.9) <sup>†</sup>	15.4 (0.9)*
D. Shale JP-5	6	30	900	14.0 (0.7)	16.7 (1.1)*
E. Decane	6	28	7500	10.3 (0.8)	9.5 (0.5)

<sup>†</sup> Mean (standard error)

\* Significantly different from control values (p < 0.05)

TABLE 6. CHANGES IN URINARY GAMMA-GLUTAMYL TRANSPEPTIDASE (GGT) ACTIVITY OF MALE RATS AFTER EXPOSURE TO HYDROCARBON VAPOR

HYDROCARBONS	GROUP N	EXPOSURE DAYS	VAPOR CONCENTRATION (mg/m <sup>3</sup> )	URINE GGT (IU/mg creat.)	
				CONTROL	EXPOSED
D. Shale JP-5	6	30	900	0.71 (0.09) <sup>†</sup>	1.40 (0.21)*
E. Decane	6	0	7500	0.39 (0.05)	0.40 (0.06)
E. Decane	6	19	7500	0.39 (0.04)	0.55 (0.06)*
E. Decane	6	28	7500	0.36 (0.03)	0.49 (0.06)

<sup>†</sup> Mean (standard error)

\* Significantly different from control values (p < 0.05)

## RESULTS

1. Consistent elevation of serum creatinine in rats exposed to 500 mg/m<sup>3</sup> of JP-5 for 80 days.
2. Decrease in creatinine clearance in rats exposed to 500 mg/m<sup>3</sup> of JP-5 for 80 days in two of the three groups.
3. Urea clearance was significantly decreased in two of the studies.
4. Urinary GGT was significantly elevated in the two studies in which it was performed.
5. During the course of the study "C" (500 mg/m<sup>3</sup> JP-5 for 80 days), the high dose rats drank significantly more water than the control rats (25 ml/day vs 21 ml/day).

6. Urinary output was measured and increased for the high dose groups (500 mg/m<sup>3</sup> and 900 mg/m<sup>3</sup>). However, it was not increased for the decane exposure.
7. The histopathology results were negative for any kidney lesions for experiments "A" and "B". The microscopic studies of the tissues from experiments "C", "D", and "E" are not yet complete.

### CONCLUSIONS

1. Male rats exposed to hydrocarbon fuels develop renal function abnormalities after 80 days of intermittent exposures, but only minimal signs are seen after 30 days of intermittent exposures.
2. These results are consistent with the findings of Gaworski, MacEwen, and Vernot, in which histopathologic evidence of renal disease was seen in male rats after 90 days of continuous exposure to 750 mg/m<sup>3</sup> of JP-5 vapor and 300 mg/m<sup>3</sup> of DFM vapor.
3. The increase of urinary GGT excretion after 30 days of exposure may be a useful biochemical marker for following the renal disease due to hydrocarbon fuels.
4. The failure of the decane exposure to produce renal function changes after 28 days of high level exposure may be a requirement of many exposures or a continuous exposure to cause the occurrence of the renal lesion.

## MORPHOLOGICAL METHODS FOR EVALUATING PULMONARY DISEASE

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Morphological examination of the lung is an essential part of many studies designed to determine effects of inhaled materials. Light microscopy (LM), transmission electron microscopy (TEM), and scanning electron microscopy (SEM) are indispensable techniques for documenting and interpreting the response of the lung to injury. Lesions in the respiratory system caused by inhalation of noxious agents are rarely uniform in distribution because of differences in concentration of the material at different locations, variation in degree of protection provided by mucus or other surface coats and regional differences in inherent sensitivity of component cell populations. Therefore, random tissue sampling techniques for morphological evaluation can yield misleading results.

The purpose of this presentation is to describe methods that permit examination of the same level of an airway and adjacent parenchyma by LM, SEM, and TEM. Using these correlated histologic and ultrastructural techniques, it is possible to detect very subtle effects and provide the most discriminating information relevant to pulmonary toxicity.

After appropriate experimental exposure, lungs are fixed by airway perfusion with Karnovsky's fixative at a constant perfusion pressure of 30 cm of water. The perfusion apparatus should allow a constant perfusion pressure regardless of the level of fluid in the container.

Using a dissecting microscope, airways are bisected longitudinally. One half of each specimen is processed for LM and TEM; the complementary half is processed for SEM.

Lung slices for LM and TEM are embedded in large blocks (16 mm x 12 mm x 5 mm) of Epon-Araldite. Blocks are cut at a thickness of 1  $\mu$ m using a Sorvall JB<sub>4</sub> microtome and glass knives. Sections are stained with toluidine blue, examined, and photographed by LM. Areas for TEM are selected by examination of 1  $\mu$ m sections.

Complementary halves of lung slices examined by LM and TEM are processed for SEM. The first step in this process is to dry the lung tissue in a critical point dryer, a technique that prevents distortion due to forces of surface tension. The dried blocks of lung tissue are glued to metal stubs and coated with metal or carbon in a vacuum evaporator. The metal coating permits electrical conductivity of the specimen which is necessary for SEM.

Areas selected by examination of the 1  $\mu$ m sections are isolated on the face of the large epoxy-embedded blocks by trimming away adjacent tissue and embedding media. The small "mesa" remaining is removed with a razor blade and glued to a blank Beem capsule. The specimen on the Beem capsule is cut at a thickness of 500 Å using an ultramicrotome and diamond knife. These sections are mounted on copper grids and examined by TEM.

Using these correlated techniques, it is possible to take full advantage of LM, SEM, and TEM in evaluating changes due to inhalation of materials. It is possible to concentrate efforts on the precise area of interest and to evaluate lesions that have a focal distribution.

Using light and transmission electron micrographs, grid overlays, and appropriate statistical procedures, it is possible to analyze quantitatively many structural characteristics. Volume fraction of the parenchyma, surface density of air spaces, capillary surface area and volume, and thickness of the air-blood barrier are among the most useful criteria to estimate. Using these data, diffusion capacity can be estimated, thus correlating structural and functional changes.

The following references describe many of these techniques in detail:

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KARNOVSKY'S FIXATIVE (1:4.5) (Approx. 550 mOsm)  
(J. Cell Biol., 27:137A, 1905)  
Modified from J. A. Nowell 3/13/72 and 3/21/69

Materials:

Paraformaldehyde (Mallinckrodt or Eastman)  
50% glutaraldehyde (Fisher biological grade)  
Cacodylic acid (Sigma)  
Calcium chloride (Anhydrous)

To Make:

16,500 ml dilute fix

Buffer (0.2M)

Cacodylic acid	192 g	or	384 g
Distilled water qs	6,000 ml		12,000 ml
	(Need 2 batches)		

Fixative (Stock)

Mix: Paraformaldehyde	120 g
Distilled water	1,500 ml
Cacodylate buffer	900 ml

Heat to 70°C in a 4 liter Erlenmeyer flask in a water bath, swirling intermittently until clear. Cool to room temperature.

Add: 50% glutaraldehyde	300 ml
Calcium chloride	1.5 g
Cacodylate buffer	300 ml
	<hr/>
	3,000 ml Total

Dilute to 1:4.5

Stock fixative	3,000 ml
Distilled water	3,000 ml
Cacodylate buffer	10,500 ml (All but 300 ml of buffer remaining from the 12,000 ml made)

Adjust to pH 7.2 ( $\pm 0.1$ ) with 1.0 N HCl (requires 90-110 ml).  
(Approx. 9.0 ml conc. HCl distilled H<sub>2</sub>O qs 100 ml)

Store at 4°C.

Filter before use.

This modification was used by Nowell, Tyler et al. in:

"Techniques for Scanning Electron Microscopy of Pulmonary Tissues and Replicas", in Proceedings of the VIIth International Congress on Electron Microscopy, Grenoble, France, 1970, 477-478.

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### SESSION III

#### GENERAL TOXICOLOGY

##### CHAIRMAN

LCDR Morris J. Cowan, Jr., MSC, USN  
Officer-in-Charge, Toxicology Detachment  
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CRITERIA USED IN ESTABLISHING ACCEPTABLE SHORT-TERM  
EXPOSURE LIMITS FOR AIR POLLUTANTS

Gordon W. Newell, Ph.D.

Associate Executive Director  
Board on Toxicology and Environmental Health Hazards  
Assembly of Life Sciences  
National Research Council/National Academy of Sciences  
Washington, D.C.

INTRODUCTION

Sixteen years ago, the Committee on Toxicology of the National Research Council published its first document for establishing emergency inhalation exposure limits, applicable to military and space chemicals (National Research Council, Committee on Toxicology, 1964).

Five years later, the National Air Pollution Control Administration and the Environmental Protection Agency published a number of criteria documents addressing the question of public exposure to a wide variety of air pollutants. In most cases, a considerable body of information had been assembled to provide the best possible basis for the recommendations involved in short-term or long-term exposures.

The format of these documents varied in detail, but conformed to a general pattern: sources, occurrence, formation, disposal, and uses were considered as background; effects on man and animals formed the basis for determining permissible exposures while effects on vegetation and materials, and significant impact on ecology or the biosphere, were noted as potentially indicating the desirability of more stringent controls.

During this period, the National Institute for Occupational Safety and Health produced numerous criteria documents on substances encountered in the workplace. Using its 1964 document as a guideline, the Committee on Toxicology has, for several years, been recommending short-term exposure limits for chemicals of concern to various federal agencies.

Attitudes about acceptable exposure conditions for both occupational and general ambient air pollutants have changed substantially since the original short-term guidelines were issued by the Committee on Toxicology. Members of the present committee felt it necessary to re-assess and revise the criteria for short-term exposures to air pollutants. This paper summarizes the results of that effort. The longer, detailed document is entitled, "Criteria for Short-Term Exposures to Air Pollutants", and was published in 1979 by the Committee on Toxicology, National Academy of Sciences, Washington, D.C.

Previously, the Committee issued separate guidelines for military and space personnel and for the general public. The present committee believes that a single set of criteria is sufficient for establishing short-term exposure limits for both the general public and more narrowly defined populations at risk, such as military or space personnel.

#### DEFINITION OF SHORT-TERM EXPOSURE

For the purpose of this paper, a short-term exposure is defined as a single exposure, usually lasting 60 minutes or less, but never more than 24 hours. For evaluation of 24 hour exposure limits, the variation and concern over shorter periods may be an important consideration. In addition to length of exposure, it is important to differentiate between repeated exposures and exposures that are exceptional and may never be repeated.

When exposures are repeated, the frequency of occurrence and the time between exposures should also be noted.

#### FACTORS TO CONSIDER IN ESTABLISHING SHORT-TERM CONCENTRATIONS OF POLLUTANTS

Various types of evidence should be assessed in reaching a decision on permissible short-term exposures:

- Results of experimental exposures in animals or humans;
- Observations on the effects of exposures to individuals;
- Epidemiology studies;
- Effects on vegetation and livestock;
- Long-term effects of short-term exposure;
- Interactions of pollutants.

Inhalation experiments with animals provide essential, preliminary information about possible effects in man, in the concentrations in which these effects may occur. They are useful in identifying adaptations that may occur with repeated exposure and enable hypotheses about the mechanisms of action of pollutants to be tested. They offer an opportunity to explore interactions between pollutants that may affect toxicity.

While human studies provide useful information of dose-response relationships for limited exposures to specific pollutants, they also may provide insights into the mechanism of effects. Human experimentation is, however, seriously limited by ethical considerations to exposures that are readily tolerated and caused only reversible effects.

The extent to which animal experiments provide quantitative data on dose-response relationships that can be reliably extrapolated to humans is debatable. Such data depend on the species used and relevance to the pharmacokinetics in both animal and man. When data from animal testing are used to predict the effects of a substance on humans, a major uncertainty in the prediction is the extrapolation from a genetically homogeneous group of experimental animals

to the genetically heterogeneous human population which contains sub-populations with varied susceptibility. There also are uncertainties with respect to the similarities between chamber exposures and actual exposures.

Observations made by the public and careful observations by scientifically trained individuals can be most valuable in the detection of functional changes that result from short-term exposures to pollutants. Unfortunately, the concentrations involved in such exposures are usually uncertain or completely unknown.

Experimental exposures and general observations are made primarily on individuals, while the focus of epidemiologic studies is on groups of people. Monitoring day-to-day concentrations of air pollutants and relating these concentrations to day-to-day morbidity or mortality responses is a useful way of conducting certain epidemiology studies.

Other epidemiologic indices useful in identifying short-term adverse affects from pollutants include such factors as sickness and absence from school or work because of illness precipitated by air pollutants, transient changes in lung or other organ functions, or development of symptoms or function impairment due to pollution. Effects of air pollutants on vegetation and livestock should not be overlooked since there are numerous examples of plant and animal sensitivity to air pollutants that do not appear to affect humans. Many plants are readily damaged by traces of ethylene, which has no effect on humans or animals. On the other hand, grazing animals, especially sheep and cattle, are severely affected by airborne arsenic, lead, and molybdenum particles that are deposited on forage and then ingested. Data on the likelihood of long-term health implications from brief exposure to chemicals are sorely lacking.

We must be reminded frequently that exposures of the public to atmospheric pollutants seldom involve a single material. The effects of a single pollutant involved in a short-term episode may be modified by interactions with one or more additional pollutants. The interaction may be physical, as in absorption of gases on solid particles, or chemical, as in photochemical smog.

### ESTABLISHMENT OF EXPOSURE LIMITS

#### CRITERIA USED TO ASSESS ACCEPTABLE EXPOSURE

- Mortality
- Morbidity (Illness)
- Physiological Measurements
  - Respiratory
  - Neuropsychologic
  - Eye Effects
  - Biochemical-Enzyme-Immunological Changes

In establishing exposure limits for chemicals, use of reliable dose-response data from human exposure is a method of choice and such data should be obtained and used whenever possible. However, human data generally are limited to accidental exposure to high concentrations of chemicals and experimental, general, and epidemiologic observations. Accidental exposure and general observations

often do not provide sufficient quantitative data to examine dose-response relationships. In addition, few epidemiologic studies are available which demonstrate effects from short-term exposure to chemicals.

When applying human data obtained from individuals or specific population groups to the general population, one must take into account the variations in genetic and health state among humans that may affect or influence their susceptibility to air pollutants. Application of safety factors and statistical estimates of risk can be used to take into account the range of human specifications.

The data necessary to evaluate the relationships between exposure to a pollutant and its effects on the population at risk are not always available from human experiences. For many air pollutants, further study in animals will be needed, and the data should be derived from at least two species. Extrapolation of the information derived from animal experiments requires experienced scientific judgement in a variety of disciplines. The evaluation should consider the conditions under which the data were obtained and, in particular, the relevance of the conditions to human exposure. How similar are the test species and the test organ and morphology, sensitivity of response to the pollutant, and in metabolism and disposition of the pollutant to man and the corresponding human organ? Development of short-term exposure limits requires that animal data be translated in a quantitative way to the human response. The response of the species most representative of man, considering both toxicologic and pharmacokinetic characteristics, should be used for determination of the appropriate exposure limit. If data are not available on which species best represents man, it is prudent to use data from the most sensitive animal model to set appropriate limits.

Safety factors have had considerable use in the past when an estimate of the maximum no observed adverse effect dose was available. The preferred term now is an "uncertainty factor", which represents the level of confidence that is justified on the basis of the animal and human toxicity data!! In the application of uncertainty factors, an estimate can be made on the basis of the lower range of the most susceptible humans. Those at unusual risk may include persons at either extreme of age, of particular nutritional state, with pre-existing disease, with hereditary susceptibility, or even at greater risk because of unusual physical exertion.

The draw-back of the uncertainty factor method is that the factor usually must be chosen without recourse to precise data on most of the above estimates of susceptibility. Attempts to estimate risks on a precise, quantitative basis have resulted from the pioneering work of Mantel and Bryan (1961) in carcinogenesis. This procedure used a probit model to extrapolate from an upper boundary on observed dose-response data to estimate the dose corresponding to a specified degree of risk or to estimate the risk at a specified dose below the experimental range (Brown, 1976; Cornfield, 1977; Mantel et al., 1975).

The approach to establishing short-term exposure levels is judged on a substance by substance basis. Where there are reliable human data, the animal data are sound, and the degree of confidence is high that irreversible effects from a single exposure will not occur, then the Committee may establish exposure limits directly from the available data; but when there are little human data, while the

animal data are sound, and it is unlikely that irreversible effects will occur from a single exposure, an uncertainty factor may be employed, the magnitude of which will depend on the quality of the data. When the evidence suggests that cancer or some other irreversible effect may occur, the Committee may then choose to provide an estimate of the level of risk of the effect occurring rather than recommending an exposure limit.

### SELECTION OF STANDARDS

For the short-term single exposure situation, the Committee has developed three definitions as follows:

SHORT-TERM PUBLIC LIMITS (STPLs) are those which relate to predictable single exposures, usually lasting 60 minutes or less, but never more than 24 hours. The STPLs are limits that, if not exceeded, are expected to be associated with only a remote possibility of a nonincapacitating reversible effect in an exposed population.

These limits differ from air quality standards in that STPLs are intended for infrequent exposures. Such an effect is one that will not cause a period of disability; will not impair one's vision, visibility, judgement or ability to breathe; and will not interfere with one's ability to escape from the site of exposure.

SHORT-TERM PUBLIC EMERGENCY LIMITS (SPELs) are those that relate to unpredictable single exposures usually lasting 60 minutes or less, but never more than 24 hours, and whose occurrence is expected to be rare in the lifetime of any one individual. SPELs reflect an acceptance of the statistical likelihood of the occurrence of a nonincapacitating reversible effect in an exposed population. They are designed to avoid significant decrements in performance during emergencies and might not contain an uncertainty factor. The acceptable level of risk will depend on the specific compound in question and the type of effect produced.

EMERGENCY EXPOSURE LIMITS (EELs) differ from SPELs only in that they apply to defined occupational groups, such as military or space personnel, rather than the general public. Such populations are more narrowly defined than the general public, and are generally younger and healthier. An EEL for a specific chemical may differ quantitatively from the SPEL for the same substance.

### ACCEPTABLE RISKS

A risk to health must be appraised from the view of establishing what, if any, is an acceptable effect. Two of the important factors to be considered are the duration and frequency of an exposure. Severe or permanent disability cannot be tolerated. Even a minor effect, such as a mildly unpleasant odor or slight irritation of the eyes and nose, if it occurs frequently, can become sufficiently objectionable to be considered in the selection of short-term exposure limits.



Of all the risks associated with the release of a pollutant, the concentration that carries the least risk to health is selected as the upper limit of the concentration of the pollutant for a short-term exposure.

### SUMMARY

Exposure limits recommended by the Committee on Toxicology differ in their intent from those developed by the American Conference of Governmental Hygienists (1978), the Occupational Safety and Health Administration (1971), the National Institute of Occupational Safety and Health, and the Environmental Protection Agency (1977).

The Committee limits apply to rare or infrequent exposures of short duration and are not applicable to the routine exposure situations encountered in industrial settings or in the ambient air.

Other limits previously described assume long-term exposure to chemicals, whether in the workroom or ambient air under normal circumstances. In addition, because the emergency exposure limits are established for narrowly defined populations, they should not be used as guidelines for all occupational groups.

The Committee on Toxicology limits are designed to be of help in planning, to assist in advance preparations for emergencies in which chemicals are released to the atmosphere, to assist in establishing procedures for handling and using chemicals, and to assist in site selection for installations with regard to their proximity to residential areas.

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## TOXICOLOGICAL EVALUATION OF AN AEROSOL OF DIESEL FUEL #2\*

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### INTRODUCTION

An aerosol generated from diesel fuel is currently being used by the military as a visual obscurant. Because there is a potential for human exposure, a series of inhalation experiments on the toxicity of the aerosol in animals are being conducted in our laboratory.

Information on adverse human health effects is minimal. A report prepared by FMC Corporation for the Department of the Navy (Hogan, P. M., personal communication) indicates that personnel exposed to this smoke screen for about 15 minutes have significant evidence of irritation of the mucus membranes. The signs include increased lacrimation with a burning sensation in the eyes, irritation of the respiratory tract frequently resulting in coughing episodes, sensitization of the skin, and occasional dizziness. When the exposure period is increased to one hour, effects on the central nervous system appear to be more predominant. These include drowsiness, poor coordination, convulsions, and vomiting. Although no references are cited, the FMC report indicates that prolonged exposure may result in sores and cataracts due to the topical action of the smoke, while subtle changes in morphology of liver, kidney, heart, and blood might result from the inhaled smoke.

While reports on the adverse health effects to humans appear to be mainly speculative, there also appears to be a dearth of information on toxicity in animals. Keen (1968) reported that exposure of rats to an aerosol of up to 10 mg/liter for unspecified periods of time did not cause any damage to the pulmonary surfactant layer as determined from pressure-volume curves of lungs. However, when Samal et al. (1975) exposed rabbits intermittently to a smoke aerosol generated from a mixture of diesel fuel and motor oil (100:6) for 2 hours per day over 80 days, antibody formation, following subcutaneous injections of typhoid-paratyphoid vaccine, was compromised.

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\* Sponsored by the U. S. Army Medical Bioengineering Research and Development Laboratory and the Office of Health and Environmental Research, U. S. Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation.

With so little information derived from the literature, we set two primary research objectives. The first was to characterize, both physically and chemically, the aerosol used for inhalation exposures. This objective includes comparison of the diesel fuel and its aerosol generated in the laboratory with similar samples obtained under field conditions, if possible. The second objective was to assess selected biological parameters during and after acute and subchronic exposures of rodents, under conditions which can be related to potential human exposures. Therefore, daily 8-hour exposures are not being conducted. Instead, the concentration, duration, and frequency of individual exposures are being widely varied to help establish their relative importance in toxicity.

Our approach has been to devote considerable effort to the development and characterization of the aerosol before beginning animal exposures. After this characterization was well underway, we began range-finding exposures to help establish safe working concentrations for subsequent subchronic exposures and also to ascertain whether mortality is proportional to the product of concentration and duration of single exposures. These experiments were only recently completed. Subchronic exposures will begin shortly in which several biologic end points will be utilized, including body weight, breathing pattern, and pulmonary resistance during exposure, histology of major organs, pulmonary function tests, neurotoxicity screening tests, function of lavaged alveolar macrophages, and immunotoxicity assays.

## METHODS

### AEROSOL GENERATION

An aerosol of diesel fuel #2 is generated in the VEES (Vehicle Engine Exhaust Smoke System) on a tank by applying diesel fuel onto the hot exhaust manifold. The fuel rapidly volatilizes and is swept out by the engine's exhaust gas. Upon reaching the cooler air outside, the fuel condenses to form very small droplets, small enough to remain suspended in the air for a considerable time and at sufficiently high concentration to obscure vision.

To conduct our inhalation exposure, we attempted to mimic these conditions of aerosol generation. The generator (Figure 1) is a 1-in.-O.D. stainless steel tube approximately 1 m long. A Vycor heater fitted into one end of the generator is maintained at 600°C. The exit end of the generator is heated by a heating tape and is maintained at 350°C. These temperatures are monitored by thermocouples. The entire generator is heavily insulated. Carrier gas (we are presently using nitrogen) introduced at the rear of the Vycor heater is heated before it contacts the diesel fuel. The diesel fuel is metered onto the tip of the Vycor heater, where it is flash vaporized, mixed with the heated carrier gas (10 L/min), and carried into the exposure chamber. Concentration of the aerosol may be varied by the rate at which the diesel fuel is metered onto the generator.

The chambers themselves are designed for whole-body exposure of rodents. They are NYU style chambers constructed of stainless steel and Plexiglas, each capable of containing 45 individually caged rats. A schematic of a chamber is also included in Figure 1.

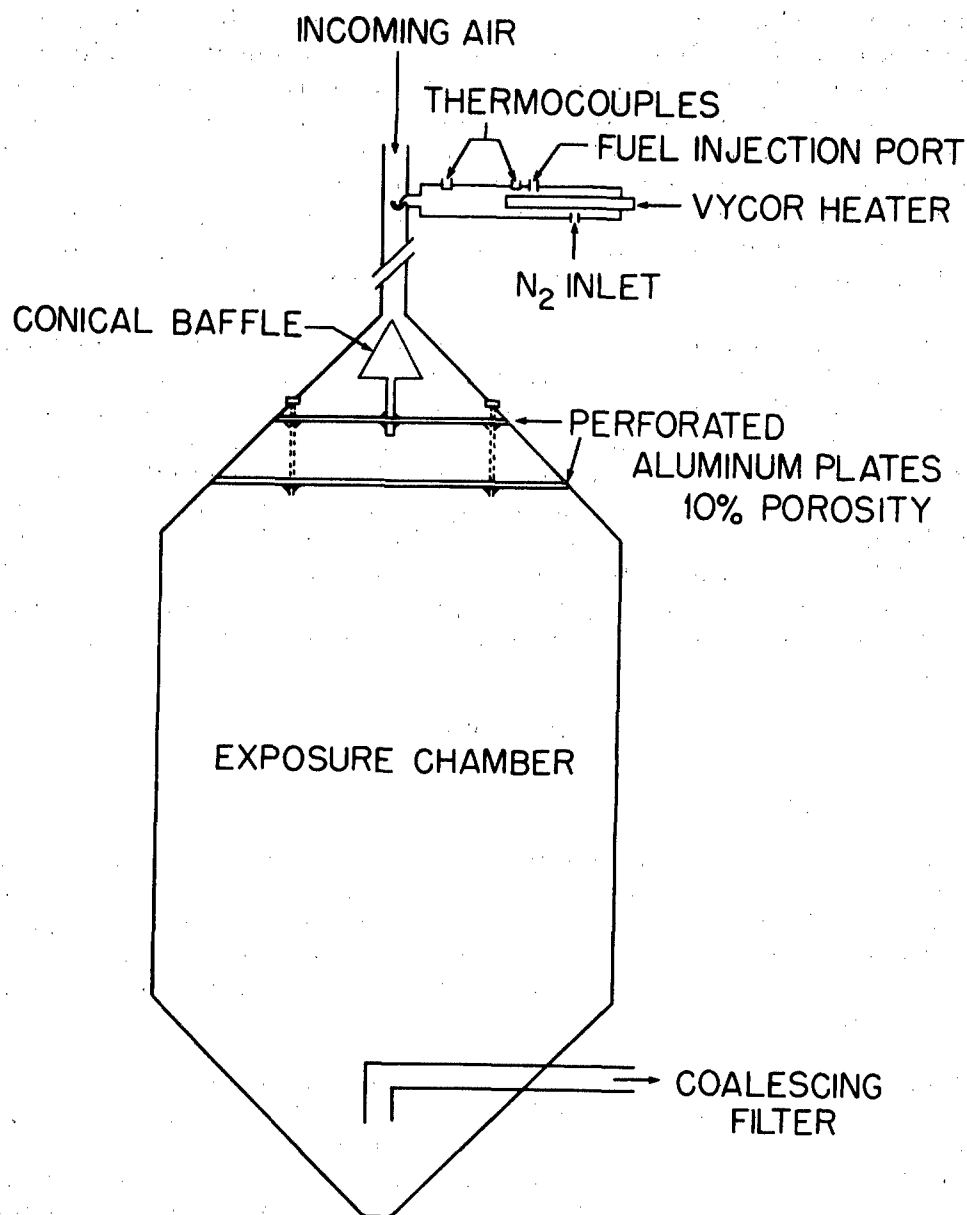


Figure 1. Schematic drawing of aerosol generator and inhalation chamber, with baffles installed.

#### AEROSOL DISTRIBUTION WITHIN EXPOSURE CHAMBER

Initially there was a problem with establishing an even distribution of the aerosol within the chamber. The incoming airstream was observed to jet straight from the inlet at the top of the chamber to the bottom of the chamber, bounce upwards off the bottom, and then return toward the bottom in a swirling motion (see Figure 2A). Since the aerosol is formed by condensation, one might anticipate

particle growth during its meandering through the chamber. Because of such potential growth and the obvious uneven distribution, we were concerned about unequal exposure of animals at different locations within the chamber. Thus, efforts were made to produce a more nearly laminar flow through the chamber.

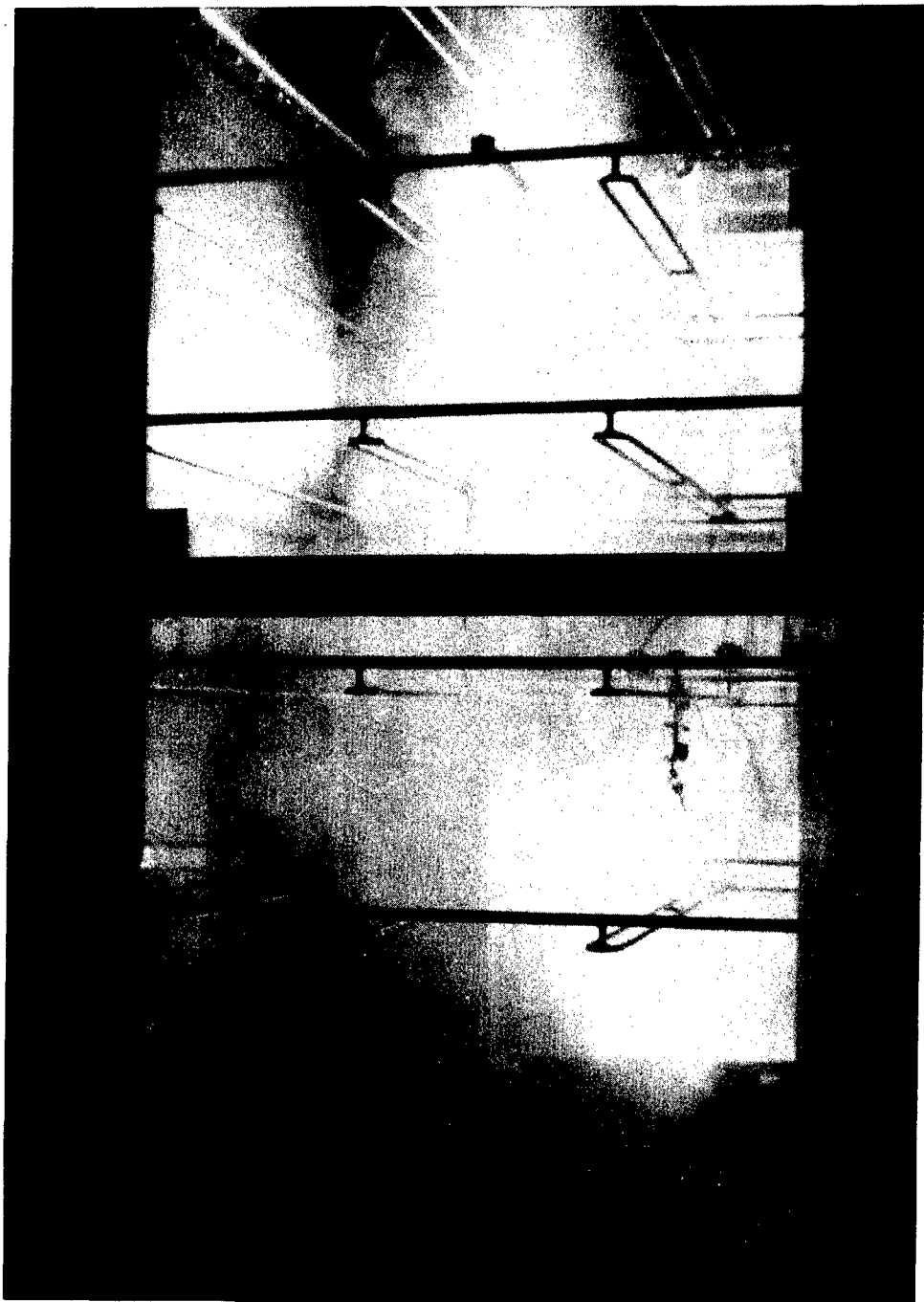


Figure 2A. Aerosol of diesel fuel entering exposure chamber without baffles in position.

A baffle system was constructed in the upper cone of the chamber to distribute incoming aerosol evenly throughout the chamber. This system may be seen in Figure 1. Incoming air first is spread across the top of the chamber's cone by a conical baffle and then slowed and further distributed across the horizontal plane of the chamber by two perforated aluminum plates with 10% porosity. Aerosol distribution throughout the chamber seemed to be much more uniform with this system installed (see Figure 2B). However, additional verification of the apparent uniformity was needed.

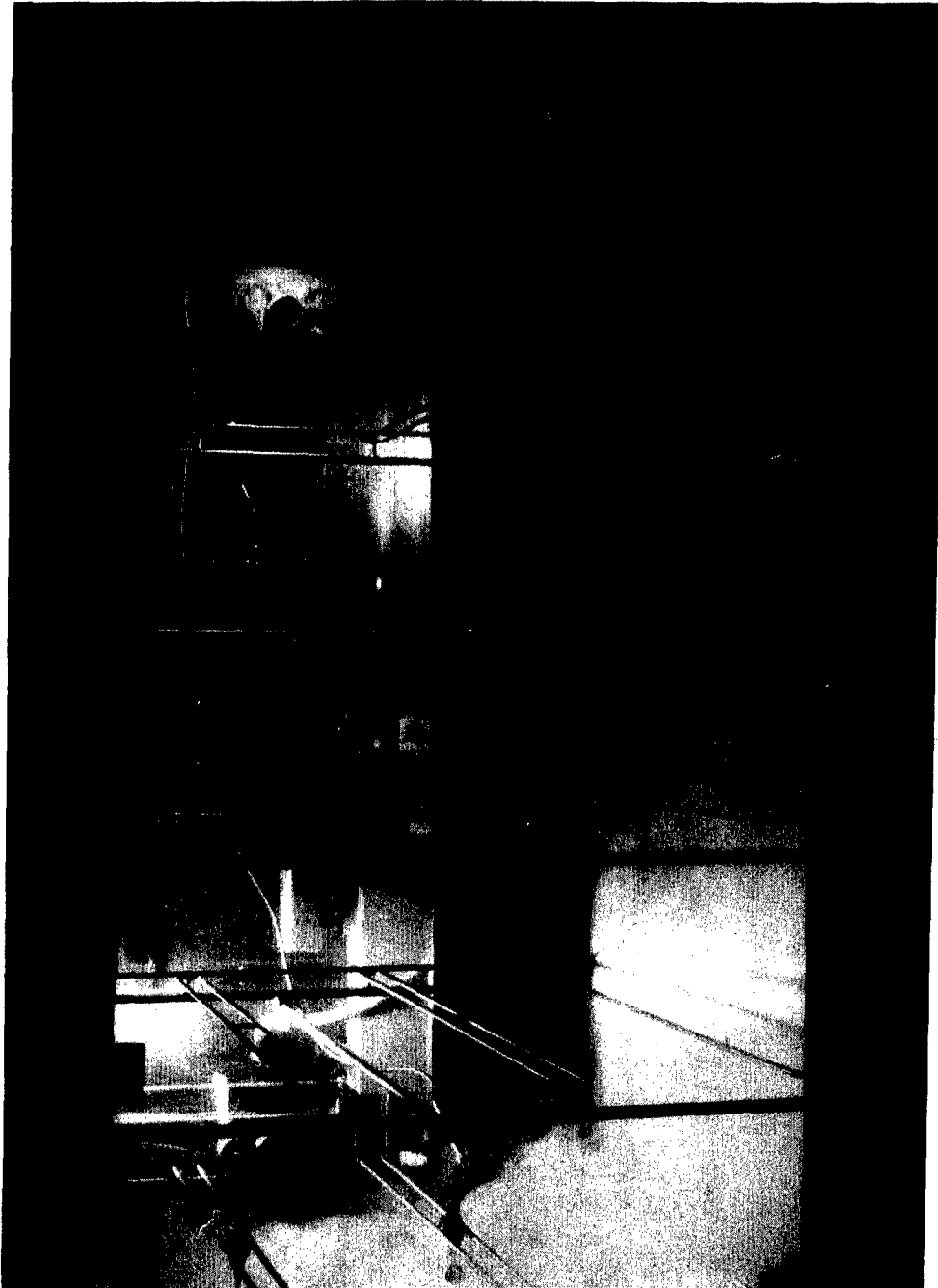


Figure 2B. Aerosol of diesel fuel entering exposure chamber with baffles in position.

Small infrared backscatter probes are routinely used to monitor aerosol concentration at several points within the chamber. These probes consist of proximity sensor chips which contain a light-emitting diode and a photo-transistor. The chips are approximately  $\frac{1}{4}$  inch in diameter and are placed on the end of a probe which may be positioned anywhere within the chamber. The chip detects the amount of light backscattered a few centimeters in front of the probe. The voltage output from the probes was adjusted so that all responded equally to the same aerosol concentration. This output was related to aerosol concentration as measured gravimetrically with Cambridge filter samples.

Series of tests were performed to investigate the uniformity of concentration on six horizontal planes in the exposure chamber (Figure 3). Aerosol concentrations during the six trials ranged from 3.1 to 3.3 mg/liter. Six infrared backscatter sensors were located on a single plane for each trial; outputs were plotted on a multipoint recorder. The records of aerosol concentrations on levels 2, 4, and 6 are shown in Figure 4. As might be anticipated, the initial increase and final decrease in concentration were more rapid at the top of the chamber than at the bottom. Concentrations of aerosol throughout the chamber were found to be uniform within approximately 10%. Gravimetric measurements of concentration at the top and bottom of the chamber also had the same amount of variability. Bearing in mind the variation normally encountered in concentration determinations, one might conclude that aerosol concentrations were uniform throughout the chamber when no cages or animals were present.

The amount of data available on aerosol concentration and particle size when animals are in the chamber is relatively limited. However, their presence affects particle size and concentration very little, if at all. Perhaps more directly related to changes in particle size (and subsequent deposition in the respiratory tract) is aerosol concentration. Since the diesel fuel aerosol is formed by coagulation, one might anticipate that particle size would be larger with higher concentrations. We observed mass median diameters of 0.5-0.7  $\mu$  with concentrations of 2.7 mg/liter as compared with mass median diameters of 1.2  $\mu$  with concentrations of 10.6 mg/liter. Intermediate concentrations have been associated with intermediate particle sizes. The influence on deposition in the respiratory tract by this change in particle size has not been determined. Studies on deposition at various sites within the lungs and airways in relation to varying concentrations of aerosol are currently underway.

#### CHEMICAL CHARACTERIZATION OF THE FUELS AND AEROSOLS

To characterize the fuel and aerosol chemically, we have employed a tiered analytical approach. That is, diesel fuel or aerosol is separated by high-pressure liquid chromatography (HPLC) followed by gas chromatographic (GC) visualization. This approach permits characterization of the fuel as to relative amounts of aromatic and aliphatic constituents and also permits visualization of the constituents according to compound class. HPLC separation is performed by isocratic elution with hexane on a 5  $\mu$  silica column. The HPLC chromatogram allows gross



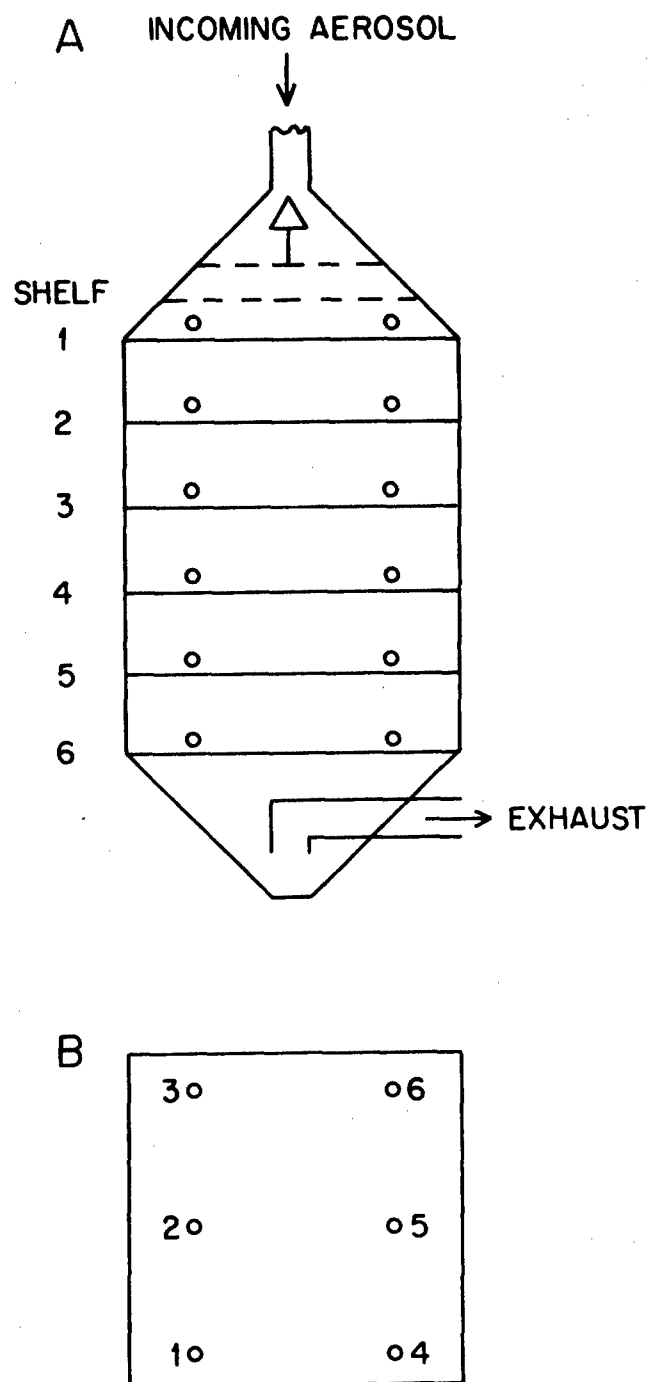


Figure 3. A, Vertical distribution of backscatter detectors on various shelves within exposure chamber. Circles indicate areas sensed by detectors. B, Horizontal distribution of backscatter detectors on each shelf.

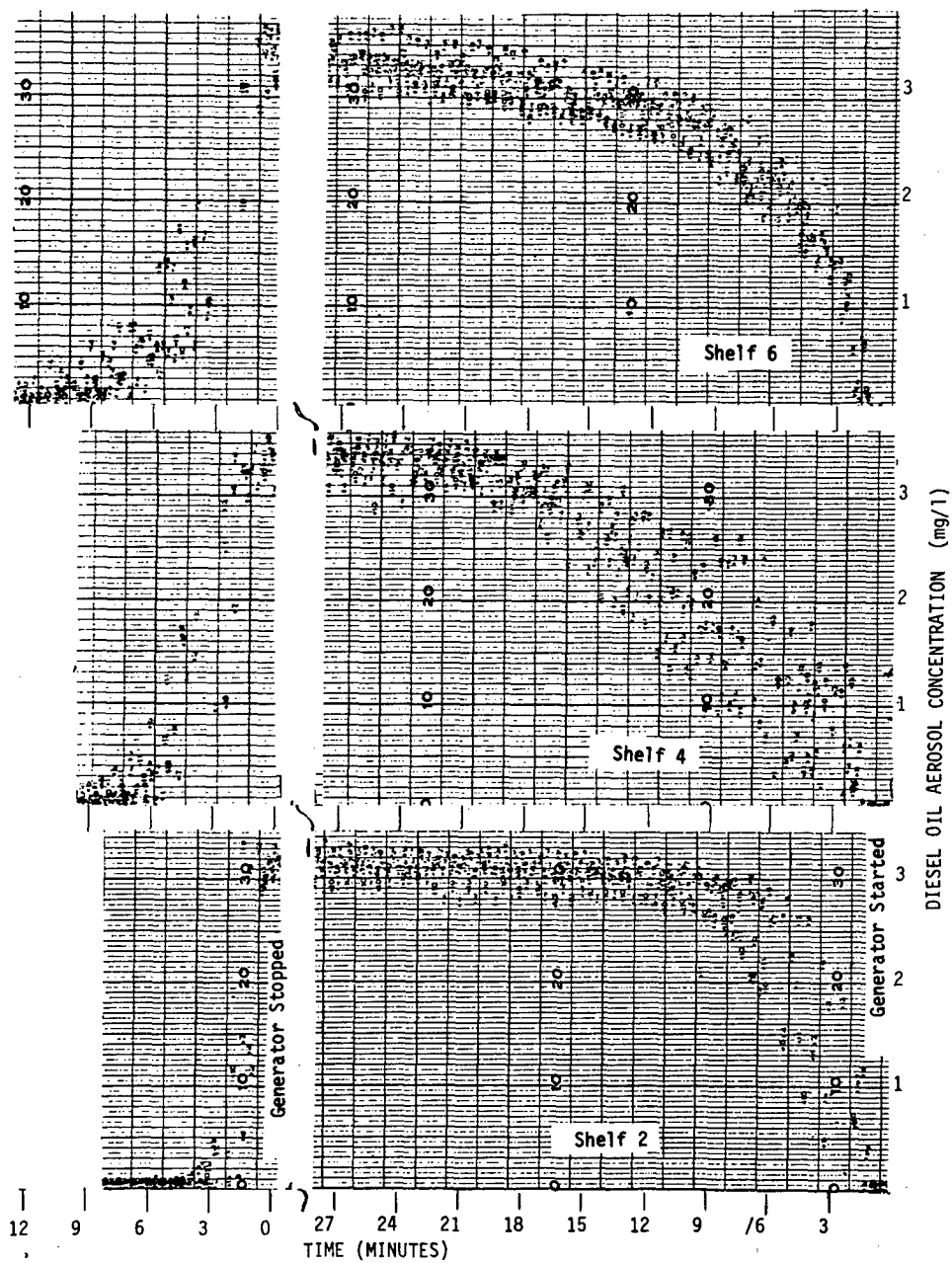


Figure 4. Aerosol concentration at three shelves in exposure chamber during initial rise in chamber concentration and decrease after generation is stopped.

comparison of differences in various fuels (Figure 5). "Phillips" denotes a standard reference fuel from Phillips Petroleum currently in use for all animal exposures. Limited samples of a random sample of commercial diesel fuel and its aerosol generated under field conditions by a tank were available to use and are referred to as the VEES samples. For the aliphatics/aromatics ratio for a specific fuel to be obtained, standardization according to refractive index sensitivity must be performed. The Phillips fuel contained 29.3% aromatics, which was considered to be in good agreement with the specified value of 30.5%.

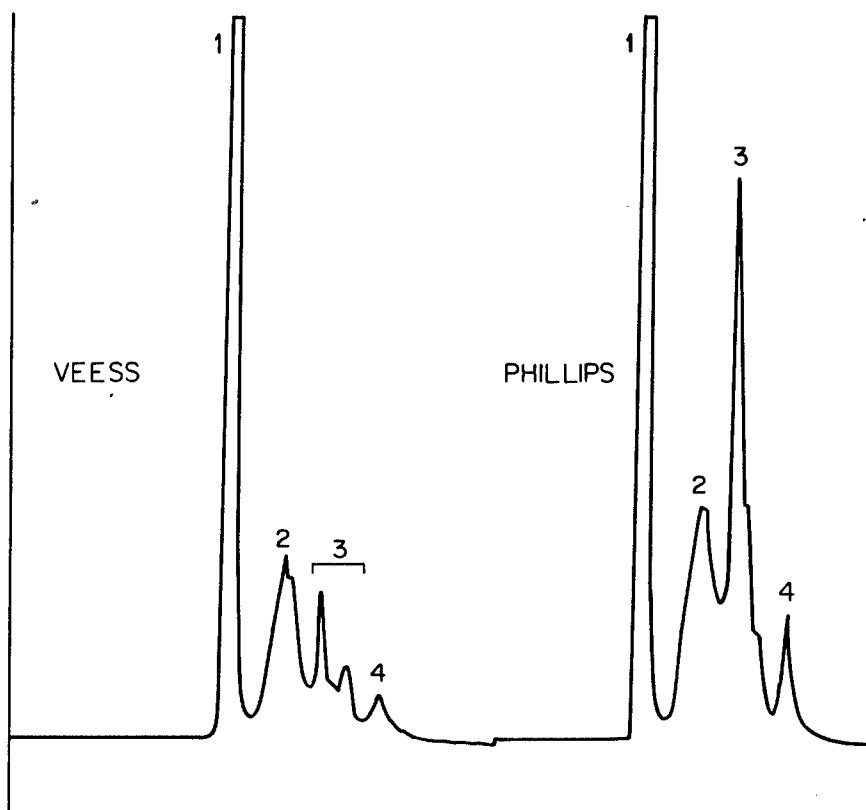


Figure 5. Comparison of profiles of VEES and Phillips fuels obtained by HPLC. Numbers refer to specific fractions (see text). Vydac column: 5  $\mu$  Alltech Ultrasphere, 9 mm i.d. x 27 cm, 50  $\mu$ l injected.

For more lucid visualization of the individual compound classes, the specific HPLC fractions are collected, reduced to a 500- $\mu$  liter volume, taken up in an appropriate amount of methylene chloride, and subjected to high-resolution GC separation. Separation is achieved on a 30-m SE-52 fused silica capillary column, with hydrogen as a carrier gas. The first fraction eluting from HPLC consists predominantly of straight- and branched-chain saturated paraffins. The second fraction is predominantly substituted benzene compounds. The third fraction is predominantly two-ring aromatics; specific constituents in this fraction include the alkylated naphthalenes, alkylated biphenyls, and small amounts of aromatic thiophenes. Major constituents of the fourth fraction include fluorene, alkylated fluorenes, phenanthrene, and alkylated phenanthrenes.

GC profiles have been obtained for the fuels and aerosols, both whole and fractionated. Through comparison of the profiles, we have addressed the question as to whether the laboratory-generated aerosol is significantly different from that present in the field. To eliminate differences which could be ascribed to differences between fuels, laboratory aerosols were generated by use of fuel sampled from an M-48 tank immediately prior to generation by the VEESS. The liquid phase of the aerosols was collected on 44-mm Cambridge filter pads. Comparison of the GC profiles of the field- and laboratory-generated aerosols (Figure 6) revealed differences primarily in the relative quantities of the more volatile constituents collected. (The peak marked "probable contaminant" in the VEESS sample is due to phthalate contamination of some of the sample bottles used.) The markedly reduced concentration of hydrocarbons eluting prior to tridecane ( $n\text{-C}_{13}\text{H}_{28}$ ) in the field samples may be due to differences in the temperatures at which the two samples were collected ( $31^\circ\text{C}$  for the field samples vs  $22^\circ\text{C}$  for the laboratory samples). In addition, the field aerosol was generated under non-steady-state conditions and most likely had considerable amounts of air drawn across the filter during periods when wind carried the smoke away from the samples. The observations suggest that the conditions of aerosol collection can influence the apparent chemical composition of the sample. However, other than these differences, changes in the gross composition of the aerosol which could be ascribed to differences of the chemical environment inside the generators were not observed. Because of the limited size of the individual samples, it was not possible to determine if alterations in trace constituent composition had occurred.

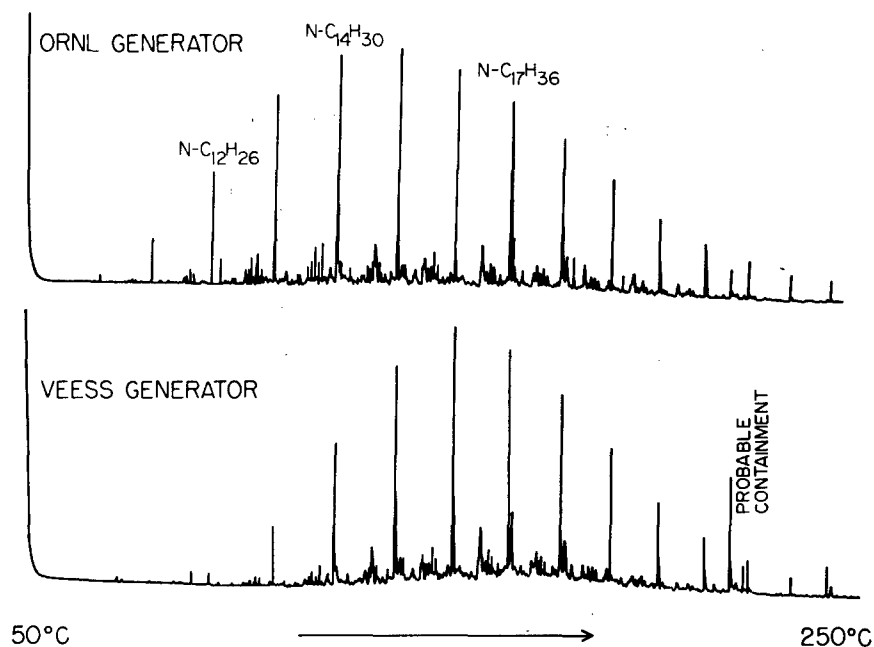


Figure 6. Comparison of GC profiles of aerosols of VEESS fuel generated in the laboratory (ORNL generator) and in the field (VEESS generator). 30-m SE-52 coated fused silica column,  $\text{H}_2$  carrier gas at 20 psi, 2  $\mu\text{l}$  splitless injection, vent after 30 sec, temperature:  $50^\circ\text{C}$ , 2-min hold,  $2^\circ/\text{min}$  increase to  $250^\circ\text{C}$ .

Also, comparison of unfractionated aerosol samples collected from the rat inhalation exposure chambers was used to determine the effect of the animals on the composition of the liquid phase of the aerosol. No selective depletion of the hydrocarbon constituents was observed.

## ACUTE TOXICITY

Acute, range-finding exposures have recently been completed. These exposures were designed to obtain an indication of: (1) concentrations of diesel fuel aerosol which might be acceptable for repeated exposure in terms of lethality, and; (2) whether mortality is related to the product of concentration and duration of exposure ( $Ct$ ) during single exposures. Each exposure group consisted of five male and five female Sprague-Dawley rats, 12-14 weeks of age. Rats were exposed for either 2, 4, or 6 hours to concentrations ranging from 2.67 to 16 mg/liter and observed for 2 weeks. After exposure, symptoms which appeared to be related to dose ( $Ct$  product) included eye irritation, loss of coordination, apparent sedation, and labored breathing. All deaths occurred within the first 48 hours after exposure, primarily from pulmonary hemorrhage and edema. Mortality data are shown in Figure 7. Statistical analysis of these data (exploratory regressions of the proportions dying, and various transforms thereof including probit, logit, and arcsine) revealed that mortality was highly related to the  $Ct$  product ( $p = 0.0001$  and 83% of the variation in mortality was explained by  $Ct$ ). The mortality response and transformations of it seemed more linear in  $\log(Ct)$  than in  $Ct$ . Estimation of the influence of exposure duration on mortality by analysis of covariance showed that mortality was slightly greater with 2-hour exposures, but: (1) exposure time accounted for less than 10% of the ability of a linear model based on  $\log(Ct)$  to explain the response, and; (2) there was no ordered relation of mortality to time. Therefore, the dominant factor in predicting response is the  $Ct$  product.

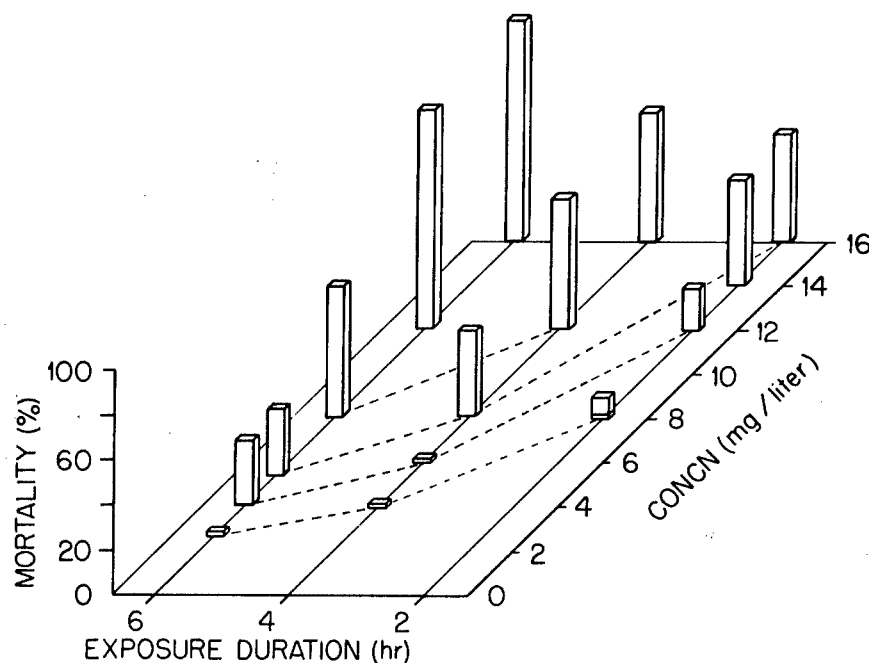


Figure 7. Three-dimensional plot showing percent mortality among groups of 10 rats after single exposures to varying combinations of concentration and duration. Dashed lines connect points of equivalent  $Ct$  products.

The next stage of the study is a series of 3-week exposures, with measurement of selected biological end points at the end of those exposures and after a subsequent 2-week recovery period following exposures. The biological end points to be tested are listed in Table 1, in addition to model compounds which have been

chosen because of their known influence of a particular parameter. These compounds have been used in the development of assays in our laboratory. In addition to the end points listed in Table 1, others to be used include refractive index of urine, body weight, food consumption, and autoradiography of the respiratory tract after injection of tritiated thymidine. The autoradiograms will be used to help identify the sites of repair (DNA synthesis) following damage to different sites within the respiratory tract after exposure to diesel fuel aerosol.

TABLE 1. MAJOR BIOLOGICAL END POINTS BEING TESTED AND MODEL COMPOUNDS USED IN ASSAY DEVELOPMENT

Parameter or Assay	Model Compound
Pulmonary resistance and breathing pattern	ozone
Pulmonary function	cadmium
Alveolar macrophage function	nitrogen dioxide
Neurotoxicity screens	triethyltin
Immunotoxicity screens	cyclophosphamide
Morphology of major organs	

Ninety-day exposures will be conducted next. The experimental design of this study will be influenced by results of the more acute repeated exposures.

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PHYSIOLOGICAL AND PHARMACOLOGICAL EFFECTS OF  
LIQUID GUN PROPELLANTS

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INTRODUCTION

The work reported in this paper was conducted in support of a request from the U. S. Army Ballistic Research Laboratory, Aberdeen Proving Ground, Maryland, for toxicity testing of monopropellants. LGP-1845 and LGP-1776 are designations for the two liquid gun monopropellants tested in this study. LGP-1845 is an aqueous mixture of 60 percent hydroxylammonium nitrate (HAN) and 20 percent triethanolammonium nitrate (TEAN). LGP-1776 consists of 60 percent hydroxylammonium nitrate and 20 percent trimethylammonium nitrate (TMAN). The three major components are shown structurally in Figure 1. These items have been recommended for large scale use in many military weapons systems and there is, therefore, the possibility of human contact in the manufacture, transportation, storage, loading, and detonation of these materials.

This study was initiated to assess the toxic mechanisms of these two mixtures and to provide a basis for advising the user of possible health hazards associated with the handling and use of these chemicals.

Previous studies performed by Naval Medical Research Institute indicated that a similar monopropellant was moderately toxic with major signs in rats and rabbits of respiratory distress and cyanosis. Basic toxicity data generated at USAEHA in the rat are shown in Table 1. The toxic signs exhibited were respiratory distress and cyanosis. Blood dyscrasias were also noted in three species and consisted of crenated cells and Heinz body formation.

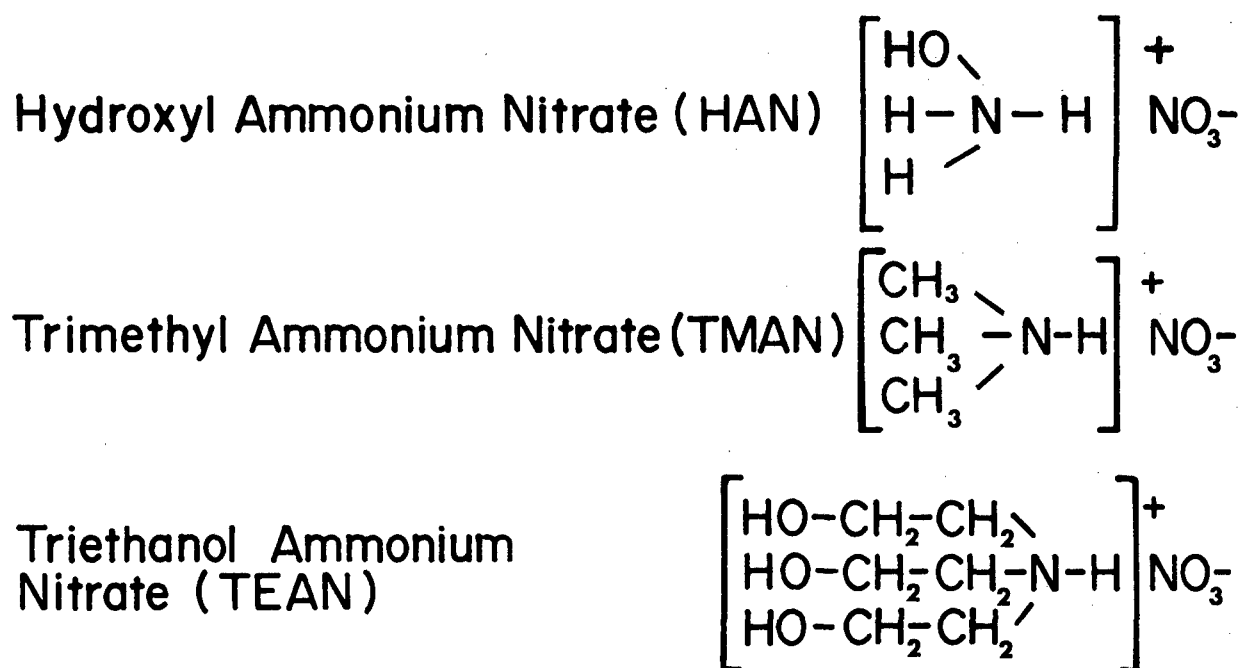


Figure 1. Components of Monopropellants.

TABLE 1. TEST RESULTS - TOXICITY OF MONOPROPELLANTS

		<u>1776</u>	<u>1845</u>	<u>HAN</u>
LD <sub>50</sub> IP mg/kg	Rats, ♂	183	202	148
	Rats, ♀	200	190	187
LD <sub>50</sub> Oral mg/kg	Rats, ♂	1060	578	653
	Rats, ♀	801	815	520
LC <sub>50</sub> Aerosol (4 hr)	Rats, ♂	>2600 mg/m <sup>3</sup>	>1600 mg/m <sup>3</sup>	
	Rats, ♀	>2000 mg/m <sup>3</sup>	>1600 mg/m <sup>3</sup>	

#### MATERIALS AND METHODS

Acute physiological and pharmacological studies were performed in beagle dogs and rats. Based on the toxic signs seen in earlier studies, emphasis was placed on monitoring the cardiovascular and respiratory systems.

All animals were examined for general physical condition and then anesthetized with sodium pentobarbital throughout the entire test procedure. The right carotid artery and the trachea were cannulated for the measurement of blood pressure and air flow. An esophageal balloon inserted at the approximate level of the thoracic inlet monitored intrathoracic pressure. A rectal thermometer monitored body temperature and needle electrodes were inserted in the standard configuration for recording the three standard electrocardiographic limb leads. Injection of methylene blue and withdrawal of venous blood samples were performed



by means of a catheter in the left femoral vein\*. Respiratory parameters were calculated with a Buxco Pulmonary Mechanics Computer and all data were recorded on a Honeywell Model 1858 fiber optic recorder.

In the dog studies serial blood samples were drawn for the determination of methemoglobin (methHb), pH, PO<sub>2</sub>, PCO<sub>2</sub>, hemoglobin, red and white blood cell counts, sodium, potassium, Heinz body formation and crenation of red blood cells. The following respiratory and cardiac parameters were monitored continuously: blood pressure, electrocardiogram, cardiac rate, intrathoracic pressure, pulmonary resistance, pulmonary compliance, tidal volume, respiratory rate and flow. The rat studies followed the same format although the only clinical laboratory parameters measured were hemoglobin, methemoglobin, and the presence of nitrites in the plasma.

Methemoglobin concentrations were determined spectrophotometrically by the method of Evelyn and Malloy (1938) as modified by Lin and Wu (1973). Calibration factors were determined by the procedure of Hawk et al. (1954). Blood gases were measured on a Corning 165/2 pH Blood Gas Analyzer, Corning Medical, Corning Glass Works, Medfield, MA.

Hemoglobin, white cell and red cell counts were obtained using a Coulter Counter, Coulter Electronics, Incorporated, Hialeah, Florida. Differential white cell counts were made utilizing the Gugol Blue Wright's Stain Kit, Summit Hill Laboratories, Avalon, New Jersey. Heinz bodies were determined by the method of Davidsohn and Henry (1969).

The Instrumentation Laboratory Flame Photometer Model 193, Instrumentation Laboratory, Incorporated, Lexington, Maryland, was used in analyzing for sodium and potassium.

## RESULTS

A total of 11 beagle dogs were utilized in this study and were dosed as follows: three with LGP-1845 (400 mg/kg); three with LGP-1776 (400 mg/kg); three with HAN (240 mg/kg); one with hydroxylamine HCl (240 mg/kg), and the remaining animal served as a control.

Prior work in rats indicated that two of the components of the liquid propellant mixtures, TMAN and TEAN, were relatively non-toxic as compared to the third component HAN. Initial investigations indicated that only HAN consistently

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\* The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Revision of the "Guide for Laboratory Animal Facilities and Care", of the Institute of Laboratory Animal Resources, National Research Council (1978). This study was performed in animal facilities fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Use of trademarked/company names does not imply endorsement by the U. S. Army, but is intended only to assist in identification of a specific compound or instrument.

produced major toxic signs and metHb. For these reasons the dog studies were limited to the mixtures 1776 and 1845 and the single component HAN.

The severe metHb resulting from the oral administration of 1776 and 1845 is shown in Figure 2. Arrows in the figure indicate the intravenous administration of methylene blue (4 mg/kg) and the resultant reduction of methemoglobin levels. Oral administration of HAN (Figure 3) produced the same results as the other two mixtures. Methemoglobin resulting from the administration of hydroxylamine, the major component of HAN, was similar to that from HAN and was also reduced by the injection of methylene blue.

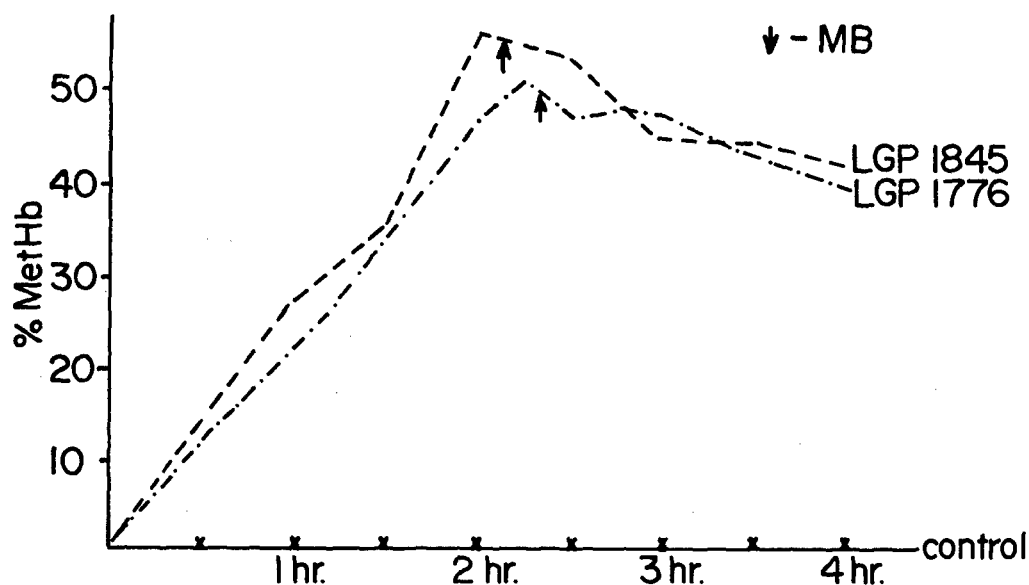


Figure 2. MetHb formation after dosage of monopropellant mixtures.

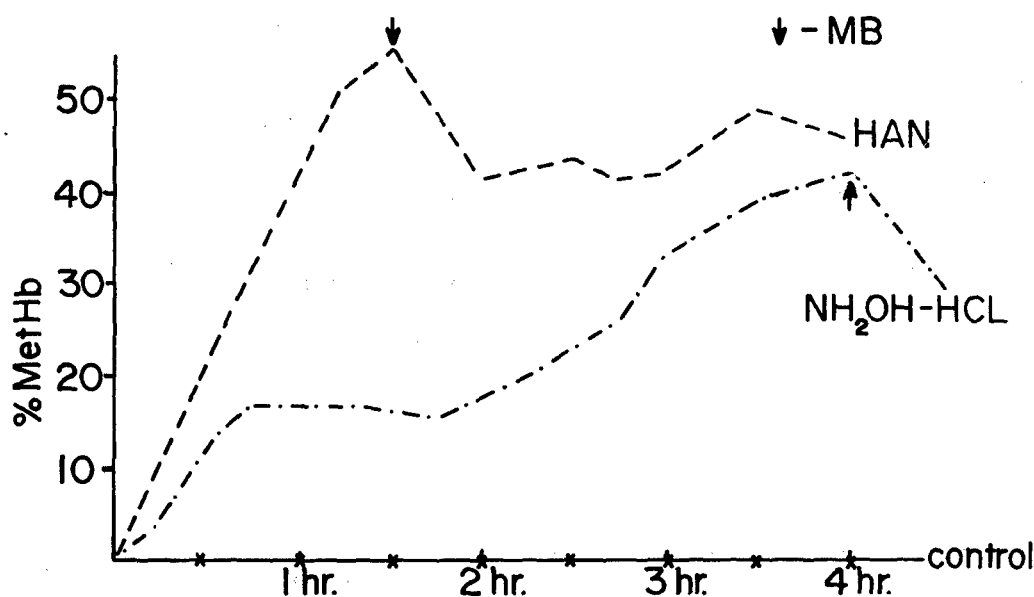


Figure 3. MetHb formation after dosage of monopropellant components.

Figures 4 and 5 show the resultant reduction of arterial oxygen tension following injection of the propellant components and indicate that although the metHb levels are reduced by the treatment with methylene blue the oxygen tension does not improve nearly as dramatically.

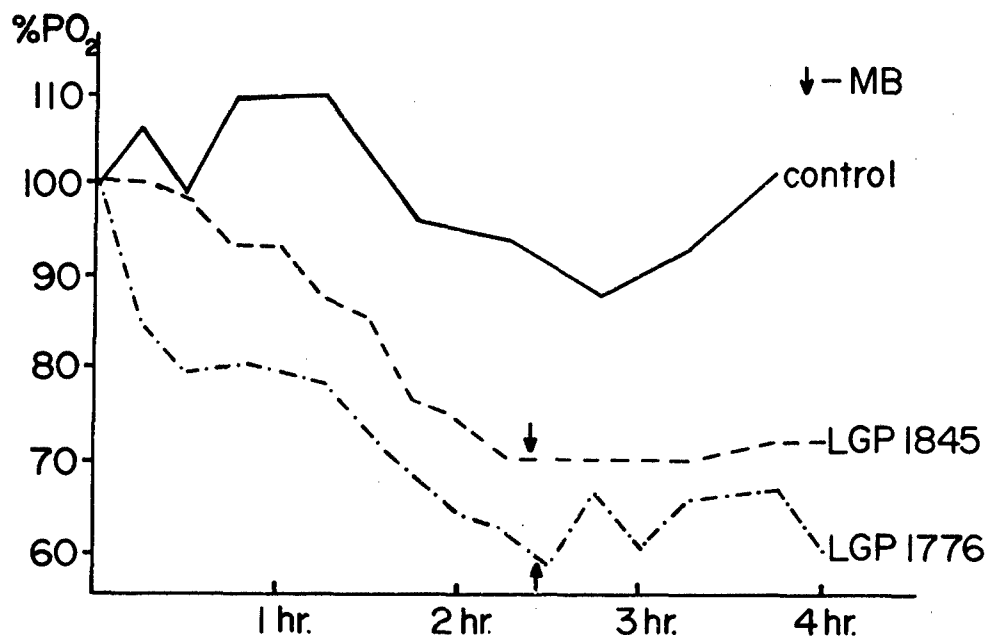


Figure 4. PO<sub>2</sub> after dosage of monopropellant mixtures.

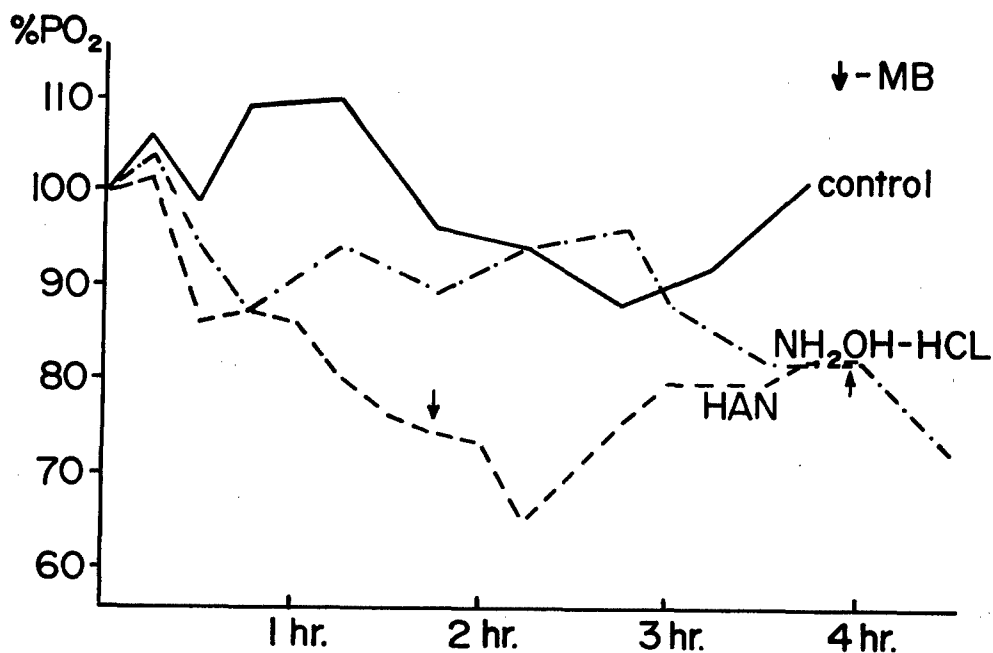


Figure 5. PO<sub>2</sub> after dosage of monopropellant components.

Intoxication by these materials also consistently produced a severe progressive drop in systemic blood pressure as shown in Figures 6 and 7. This acutely irreversible effect is presumed to be due to a severe peripheral vasodilation due to the high nitrate levels of these materials and, as is shown in Figure 7, was not seen with the hydroxylamine hydrochloride.

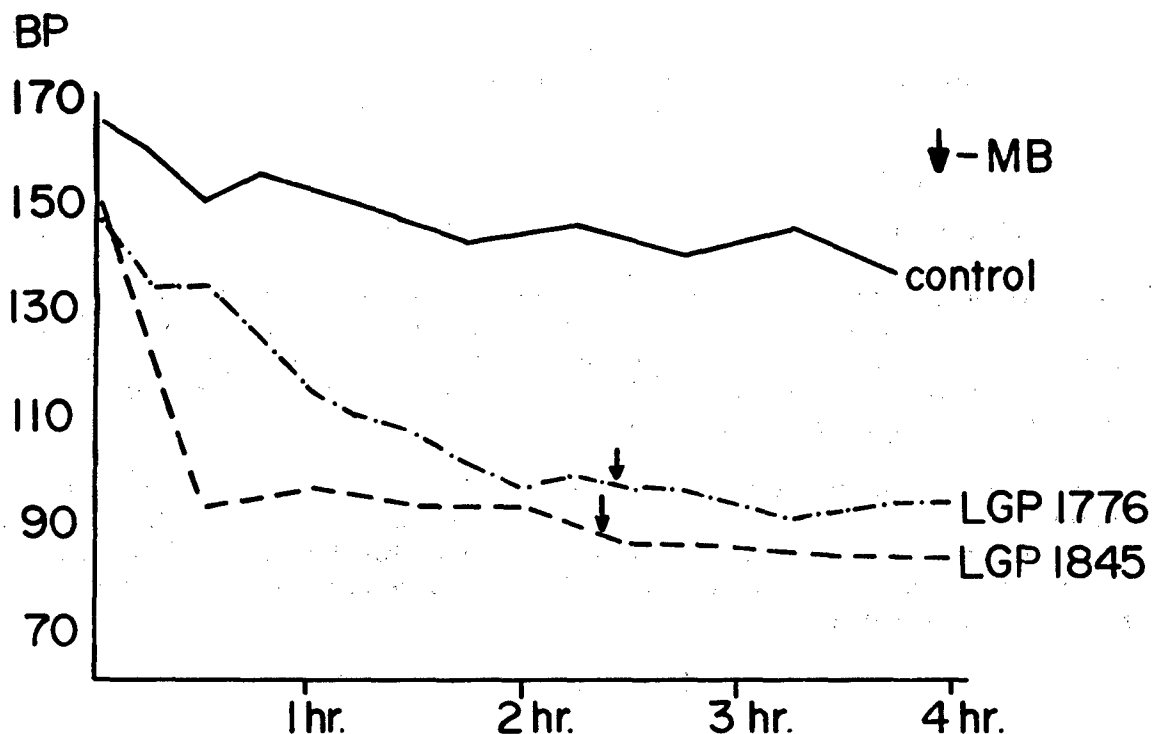


Figure 6. Mean B.P. after dosage of monopropellant mixtures.

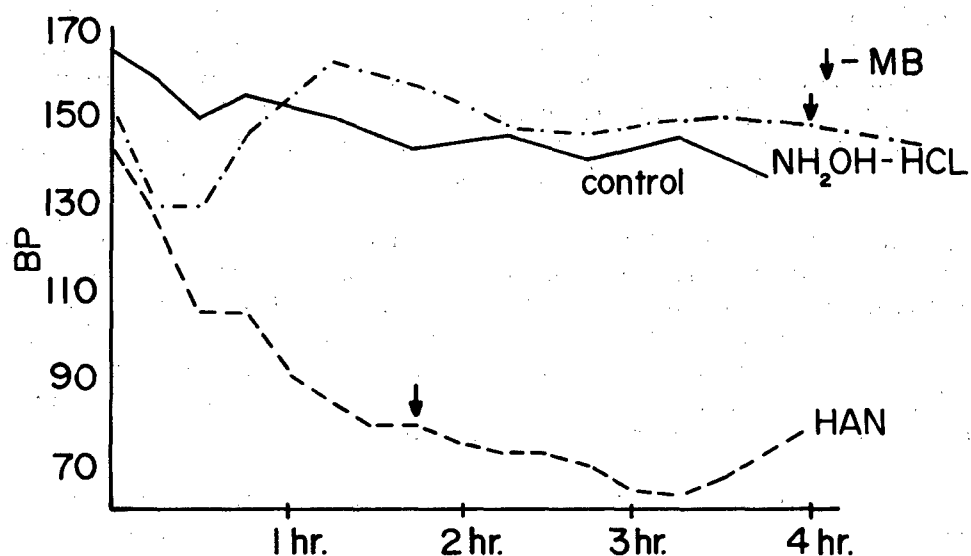


Figure 7. Mean B.P. after dosage of monopropellant components.

The toxicity encountered following oral administration of these materials is further compounded by the blood dyscrasias noted in Table 2. Crenation of the red blood cells and Heinz body formation was seen consistently with 1776, 1845, HAN and hydroxylamine.

TABLE 2. TEST RESULTS - DOGS (400 mg/kg)

Signs	LGP 1776/LGP 1845 Time After Administration (Hours)			
	0	1	2	3
Abnormal Cells	-/-	-/-	+/+	+/+
Heinz Bodies	-/-	+/+	+/+	+/+

No effects on pulmonary compliance or resistance were detected in these studies. These pulmonary function measurements did not significantly differ from either control animals or from the individual animal's preexposure values. This was confirmed by histologic examination of the lung tissue wherein no exposure related lesions were observed.

Respiratory rate increased dramatically in all dogs dosed with either the monopropellant mixtures or their components. This was assumed to be a compensatory mechanism in response to the high methemoglobin levels and lowered oxygen tension. Heart rate, however, did not change significantly through the course of the experiments.

### DISCUSSION

The formation of methemoglobin in dogs from oral administration of these nitrate containing monopropellants seemed to be in conflict with previous reports in the literature that dogs require preformed nitrite to induce methemoglobinemia. Several review articles on nitrates (Emerick, 1974; Ridder and Oehme, 1974) have quoted the study by Greene et al. (1954), that methemoglobinemia in dogs cannot be demonstrated as a result of nitrate ingestion. At first it was suspected that the nitrates in the monopropellants were being reduced to nitrites in vivo. The standard diphenylamine spot test (Householder et al., 1966) was used to assay the blood for nitrite formation. However, in retrospect, this test was proven to be positive for both nitrates and nitrites and, therefore, unreliable.

Nitrite is known to be about ten times more toxic than nitrate (Burrows, 1980). The possibility of nitrite formation from these nitrate containing compounds is still being investigated at this laboratory. Further studies have been initiated on rats using a nitrite specific test, the  $\alpha$ -naphthylamine nitrite reduction test. Preliminary results indicate that none of the monopropellant mixtures or components form nitrite in vivo. Therefore, it is assumed that the methHb formation is caused by the hydroxylamine containing component HAN.

The degree of methHb formed was species variable. The rat rapidly formed and reduced methHb whereas the dog with a less active methHb reductase system was unable to reduce the methHb formed from equivalent doses of the monopropellants. The specific lethal range of methemoglobinemia in dogs could not be determined

from the literature; therefore, in these experiments methylene blue (4 mg/kg) was given intravenously when the methHb reached 50 percent, the lower level of methHb lethality for farm animals (Jones et al., 1977).

The toxic signs observed in the dogs and rats (cyanosis, etc.) are the result of hypoxia due to decreased arterial oxygen tension caused by methemoglobinemia. The formation of Heinz bodies has no direct correlation with methHb formation according to Rentsch (1968). The increase in crenated red cells and Heinz bodies may be a nonspecific reaction to a toxic agent.

The hypotensive effect of the monopropellant mixtures may be attributed to the vasodilatory action of the nitrate portion of these compounds. This acutely irreversible drop in blood pressure was seen in both the dogs and rats.

### CONCLUSIONS

Acute studies in several species indicate that these compounds are moderately toxic and are readily absorbed dermally. The toxicity evidenced by these mixtures appears to be due to the single component hydroxylammonium nitrate.

The major toxic effects involve the erythrocyte as evidenced by crenation of cells, Heinz body formation, and significant methemoglobin production. Principal toxic signs are respiratory distress, cyanosis, and a severe drop in mean systemic blood pressure.

Additional studies should be initiated to determine if the blood dyscrasias noted are reversible and to further investigate the precise mechanism and duration of the severe hypotension seen in the acute studies.

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## TOXICOLOGY STUDIES IN SUPPORT OF SMOKE SYSTEMS DEVELOPMENT

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### INTRODUCTION

Toxicology studies are conducted at Chemical Systems Laboratory to establish a toxicologic data base for smokes to aid in selection of final candidates for advanced development by the Project Manager, Smokes/Obscurants. These studies provide the smoke systems development community with timely toxicity testing of candidate smokes.

The smoke toxicology program at Chemical Systems Laboratory is divided into four sequential phases: (1) literature searches; (2) preliminary toxicity testing; (3) acute toxicity testing, and; (4) subchronic toxicity testing. A technical review committee meets periodically to decide whether candidate formulations warrant further toxicity testing. A formulation may be rejected at any time due to its toxicity (risk/benefit), engineering considerations, logistics, cost or obscuration potential.

In the first investigative phase, literature searches are conducted to determine what appropriate toxicology data are available. The results of these searches are reviewed to determine whether the existing data are adequate and, if not, what further testing is required by Chemical Systems Laboratory.

In the second investigative phase, preliminary toxicity testing, the imminent hazards of the candidate are defined through toxicity screening tests promulgated for hazardous substances by the Food and Drug Administration, the Department of Transportation, the Consumer Products Safety Commission, and the Environmental Protection Agency. These tests are delineated in the Code of Federal Regulations, CFR 16, Commercial Practices Chapter II, CFR 49 Transportation, Chapter I, and CFR 40, No. 129, Environmental Protection Agency Part II. These tests include oral toxicity and inhalation toxicity in rats, and cutaneous toxicity, corrosivity, eye and skin irritation in rabbits. Two other tests have been incorporated into this initial test phase by Chemical Systems Laboratory: (1) the intravenous toxicity in the mouse to establish the intrinsic toxicity of the test substance for comparison to other substances, and; (2) the Ames microbiological mutagenicity bioassay which may indicate carcinogenic potential.



In the third investigative phase, acute toxicity testing is performed on formulation ingredients and the products of disseminated smokes. These tests include the determination of the median lethal dose ( $LD_{50}$ ) by the intravenous and oral routes in rats and the skin sensitization potential in guinea pigs. Inhalation toxicity studies in two rodent species are conducted to determine the median lethal dose ( $LCt_{50}$ -concentration X exposure time). In most instances attempts are made to simulate worst case exposures with the formulation as intended for field usage with field disseminators. No-effect single exposure levels are being determined. Animals are being examined for pathologic lesions in the following organ tissues: heart, lung, liver, spleen, kidney, brain, eye, trachea, nares, adrenal, stomach, bladder, gonad, pancreas, small and large intestine. The following blood parameters are evaluated in surviving animals: triglycerides, cholesterol, glucose, urea nitrogen, creatinine, alkaline phosphatase, glutamic pyruvic transaminase, glutamic oxalacetic transaminase, albumin, globulin, calcium, phosphorous, red blood cell count, white blood cell count, differential white count, hemoglobin and hematocrit. Rodents exposed to a non-lethal dose also undergo pulmonary function testing to aid in defining a non-permanent effect level. The acute inhalation studies also provide guidance for selection of doses for repeated exposure studies.

A follow-up mutagenicity study in the fruit fly is incorporated into this phase of the study. Other mutagenicity tests may also be incorporated into this phase.

In the fourth investigative phase, subchronic toxicity testing, one group of animals is exposed daily, five days per week, for up to thirteen weeks to anticipated field exposure dosages, and another group of animals to some increment of field exposure dosages. Attempts are made to establish no-effect levels and minimal-effect levels for these exposures. Again, attempts are made to simulate field exposures using field disseminating devices - thermal munitions, vaporization - condensation generators, dust dispersers, etc. As in all inhalation exposures, chemical and physical characterizations of the smoke cloud for each exposure are determined.

Usually, inbred strains of rats and mice are the species of choice. An outbred strain of rats, Sprague-Dawley, is employed in reproduction studies. The animals are observed daily for toxic signs and weighed weekly. Animals are equally divided (male and female) into two or three exposure groups and a sham exposure group (controls). Each of these groups is divided into three sub-groups: a six-week exposure group, a thirteen-week exposure group and a group to be held 30 days after thirteen exposure weeks. All of the mice and half of the inbred rats are submitted for necropsies. Hematology studies are conducted in rats and mice; blood chemistries, in rats only. Blood parameters are the same as those indicated in acute studies. Selected body organs are weighed. Animals are examined histologically: turbinates, brain, pituitary, thoracic spinal cord, eyes, salivary glands, zymal gland, thyroid, parathyroids, thymus, larynx, trachea, esophagus, lung, heart, liver, spleen, kidneys, adrenals, stomach, pancreas, duodenum, jejunum, ileum, caecum, large intestine, rectum, lymph node, urinary bladder, testes, prostate, seminal vesicle, ovaries, uterus, skin, bone with marrow (sternum), sciatic nerve, biceps femoris muscle, rib (costochondral junction), mammary gland, lesions and/or tissue masses.

Half of the inbred rats undergo cardiovascular, respiratory and behavioral testing. The following table displays the parameters measured.

#### MEASURED BIOLOGICAL PARAMETERS IN SMALL RODENTS

##### RESPIRATORY

Tidal Volume (TV)  
Minute Volume (MV)  
Breathing Rate (BR)  
TV + 6% CO<sub>2</sub>  
MV + 6% CO<sub>2</sub>  
BR + 6% CO<sub>2</sub>

##### CARDIOVASCULAR

Blood Pressure (Systolic)  
Heart Rate  
ECG - P Amplitude  
- P Duration  
- QRS Interval  
- QT Interval

##### BEHAVIORAL

Treadmill Run  
Passive Avoidance  
Reflex Activity  
Pupillary (light)  
Tail pulse (temp. change)  
Respiratory (CO<sub>2</sub>)  
Sniffing behavior

The exposure of outbred rats serve to screen the candidate smoke for its potential effects on the mammalian reproduction system with regard to dominant lethal mutations, teratogenesis, fetal toxicity, and reproductive performance.

#### VISUAL AIDS USED IN SUPPORT OF THIS PRESENTATION

TITLE: Smoke toxicology

OBJECTIVE: To perform acute and subchronic toxicologic studies that will aid in defining the hazards to man from single or repeated exposures to smoke/obscurants in user situations.

## SMOKE TOXICOLOGY

	<u>FY78</u>	<u>FY79</u>	<u>FY80</u>	<u>FY81</u>	<u>FY82</u>
Literature Searches	10*	20	15	10	10 →
Preliminary Toxicity Studies	10	3	5	5	5 →
Acute Toxicity Studies	6	5	4	4	4 →
Subchronic Toxicity Studies	2	2	3	2	2 →
Supplementary Toxicity Studies				2	2 →

\* Estimated number of candidate smokes tested.

## CHEMICAL SYSTEMS LABORATORY TOXICOLOGIC EVALUATION OF CHEMICAL SMOKE SYSTEMS

### LITERATURE SURVEY

Search: Lockheed Dialogue  
Toxline  
Medline  
Chemical Abstracts  
National Technical Information Services  
Current Contents  
Documentation of Threshold Limit Values  
Registry of Toxic Substances  
Biosis  
Toxicology Tests

### PRELIMINARY TOXICITY SCREEN

1. CFR 21 and 49 Oral, Cutaneous, and Inhalation - 2 Doses
2. Eye and Skin Irritation
3. Intravenous LD<sub>50</sub> - Mice
4. Ames Microbiological Mutagenic Bioassay

CHEMICAL SYSTEMS LABORATORY  
TOXICOLOGIC EVALUATION OF CHEMICAL SMOKE SYSTEMS

ACUTE TOXICITY PROFILE

1. Inhalation LC<sub>50</sub>
  - A. Blood Studies
  - B. Pathology
  - C. Lung Function
  - D. Toxic Signs
2. Chemical/Physical
  - A. Cloud Characterization
  - B. Analytical Methods
3. Toxicity of Cloud Condensate
  - A. Intravenous LD<sub>50</sub> - Rat
  - B. Oral LD<sub>50</sub> - Rat
  - C. Skin Sensitization
  - D. Mutagen-Drosophila
  - E. Micronucleus

SUBCHRONIC TOXICITY

90-Day Exposures

Toxicity  
Pathology  
Blood Studies  
Serial Sacrifices  
Lung Function  
Physiology  
Behavior  
Reproduction

BLOOD PARAMETERS EVALUATED

Triglycerides	Glutamic Pyruvic Transaminase
Cholesterol	Glutamic Oxalacetic Transaminase
Glucose	Albumin
Urea Nitrogen	Globulin
Creatinine	Uric Acid
Sodium	Calcium
Potassium	Phosphorous
Chloride	Red Blood Cell Count
Carbon Dioxide	White Blood Cell Count
Alkaline Phosphatase	Differential White Count
Hematocrit	Hemoglobin

## TISSUES EXAMINED HISTOLOGICALLY

Turbinates	Heart	Urinary Bladder
Brain	Liver	Testes
Thoracic Spinal Cord	Spleen	Prostate
Eyes	Adrenals	Seminal Vesicle
Salivary Glands	Stomach	Uterus
Zymbal Gland	Pancreas	Skin
Thyroid	Duodenum	Bone with Marrow
Parathyroids	Jejunum	(Sternum)
Thymus	Ileum	Sciatic Nerve
Larynx	Caecum	Biceps Femoris Muscle
Trachea	Large Intestine	Rib
Esophagus	Rectum	(Castro-Chondral
Lung	Lymph Node	Junction)
Lesions and/or Tissue Masses		Mammary Gland

## METHODOLOGY DEVELOPMENT TO MEASURE BIOLOGICAL PARAMETERS IN SMALL RODENTS

### Respiratory

Tidal Volume (TV)  
 Minute Volume (MV)  
 Breathing Rate (BR)  
 TV + 6% CO<sub>2</sub>  
 MV + 6% CO<sub>2</sub>  
 BR + 6% CO<sub>2</sub>

### Cardiovascular

Blood Pressure (Systolic)  
 Heart Rate  
 ECG - P Amplitude  
       - P Duration  
       - PR Interval  
       - QRS Interval  
       - QT Interval

### Behavioral

Treadmill Run  
 Passive Avoidance  
 Reflex Activity  
   Pupillary (Light)  
   Tail Pulse (Temp. Change)  
   Respiratory (CO<sub>2</sub>)  
   Sniffing Behavior

### Other

Reproduction  
 Teratology  
 Mutagenicity

# ACUTE TOXICITY OF WHITE PHOSPHORUS/FELT CLOUD CONDENSATE

<u>Test</u>	<u>Results</u>
Intravenous LD <sub>50</sub> in the Mouse	0.41 (0.36 - 0.48) mg/kg
Oral Toxicity in the Rat	DOSE                      MORTALITY 8.25 mg/kg                10/10 0.08 mg/kg                0/10
Intravenous LD <sub>50</sub> in the Rat	0.23 (0.19 - 0.29) mg/kg
Eye Irritation in the Rabbit	0.1 ml - SEVERE IRRITATION, CORNEAL ULCER
Skin Irritation in the Rabbit	0.5 ml - SCORE: 8.00 PRIMARY SKIN IRRITANT
Skin Sensitization in the Guinea Pig	NEGATIVE
Ames Assay	NEGATIVE
Drosophila	NEGATIVE

## COMPARATIVE LC<sub>50</sub> (MG MIN/M<sup>3</sup>) OF PHOSPHORUS SMOKES BASED ON INHALATION TOXICITY STUDIES CONDUCTED IN THREE LABORATORIES

SPECIES	WHITE PHOSPHORUS (United Kingdom)*	WHITE PHOSPHORUS/FELT (CSL)	RED PHOSPHORUS/BUTYL RUBBER (CSL)	RED PHOSPHORUS/MAGNESIUM (RALTECH SCIENTIFIC SER)
Guinea Pig	3,641 (3116-4783)	5,321 (4749-5961)	4,040 (1521-10,724)	
Rat	73,237 (56,483-89,740)	94,125 (77,200-114,764)	222,715 (192,651-257,471)	269,684 (242,048-300,476)

\* United Kingdom (UK) data was based on analysis of phosphorus pentoxide; CSL data is based on phosphoric acids. If United Kingdom results are based on phosphorus acids (Factor: 1.44), UK and CSL results are essentially identical.

SUBCHRONIC TOXICITY STUDIES IN RATS OF  
WHITE PHOSPHORUS/FELT

		15 Min Daily Exposure Dose (mg min /cu m)		
		<u>17,415</u>	<u>8,833</u>	<u>2,887</u>
Toxic Signs	40% Dyspnea and Wheezing	Negative	Negative	Negative
Pathology				
Spontaneous Deaths	Laryngitis and Tracheitis With Lesions and Blister- ing, Lung Congestion, Asphyxiation	None	None	None
Six-Week Exposure	Laryngitis and Tracheitis Pneumonia	Tracheitis Laryngitis	Tracheitis	Tracheitis
Thirteen-Week Exposure	Laryngitis	Tracheitis	Negative	Negative
Physiology	Rales and Tendency for Reduced Tidal Volume	Negative	Negative	Negative

## HEALTH EFFECTS OF SYNTHETIC FUELS

Eli J. Salmon, Ph.D.

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Washington, D.C.BACKGROUND

There is at present an imbalance between the pattern of energy resources and the pattern of energy consumption both in the United States and in the world. This is clearly illustrated in Figures 1 and 2 (Fluor Engineers and Constructors, Inc., 1979a). Whereas crude oil and natural gas accounted in 1977 for about 75 percent of the total U. S. energy consumption, they accounted for only 6.8 percent of U. S. fossil energy resources. Over 90 percent of our recoverable fossil reserves are in the solid forms. At the same time, coal accounted for only 18.6 percent of energy consumption in 1977. Nuclear, hydro, thermal, and solar energies may supply in the future increasing demands for energy. They cannot, however, supply the demand for liquid fuels which is critical for transportation. Figure 3 (Fluor Engineers and Constructors, Inc., 1979a) illustrates the increasing gap which is projected between U. S. production and consumption of liquid fuels. This gap is the main impetus toward coal conversion and oil shale retorting. Table 1 (Environmental Protection Agency, 1980b) provides a projection of the growth of the coal conversion industry during the period 1985-2000. The production of coal derived synthetic fuels is expected to increase from 0.4-0.6 million barrels a day oil equivalent (mbdoe) in 1990 to about 1.6-2.5 mbdoe in 2000. Shale oil production is expected to increase from 0.33 mbdoe in 1990 to 1.55 mbdoe by 2000 (Environmental Protection Agency, 1980b).

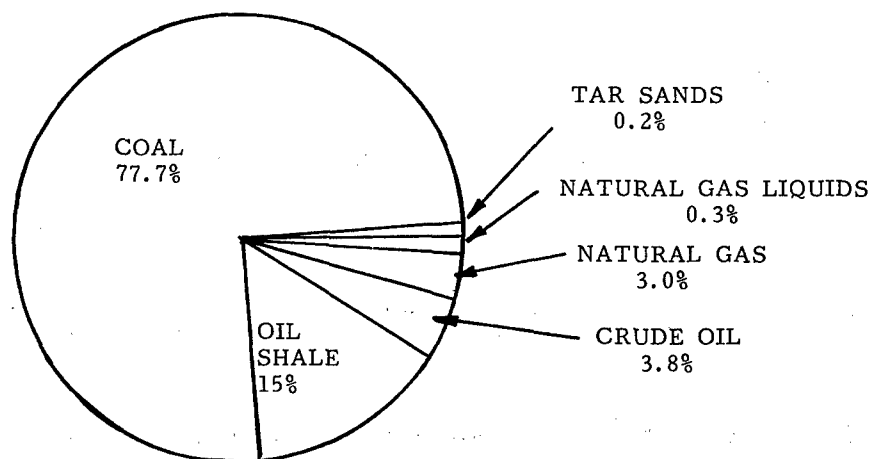


Figure 1. U. S. fossil energy resources distribution.



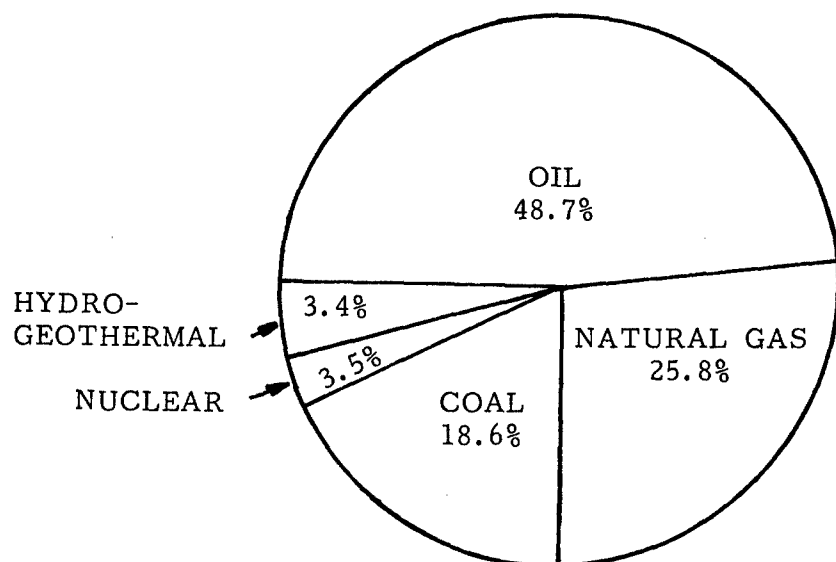


Figure 2. U. S. energy use 1977.

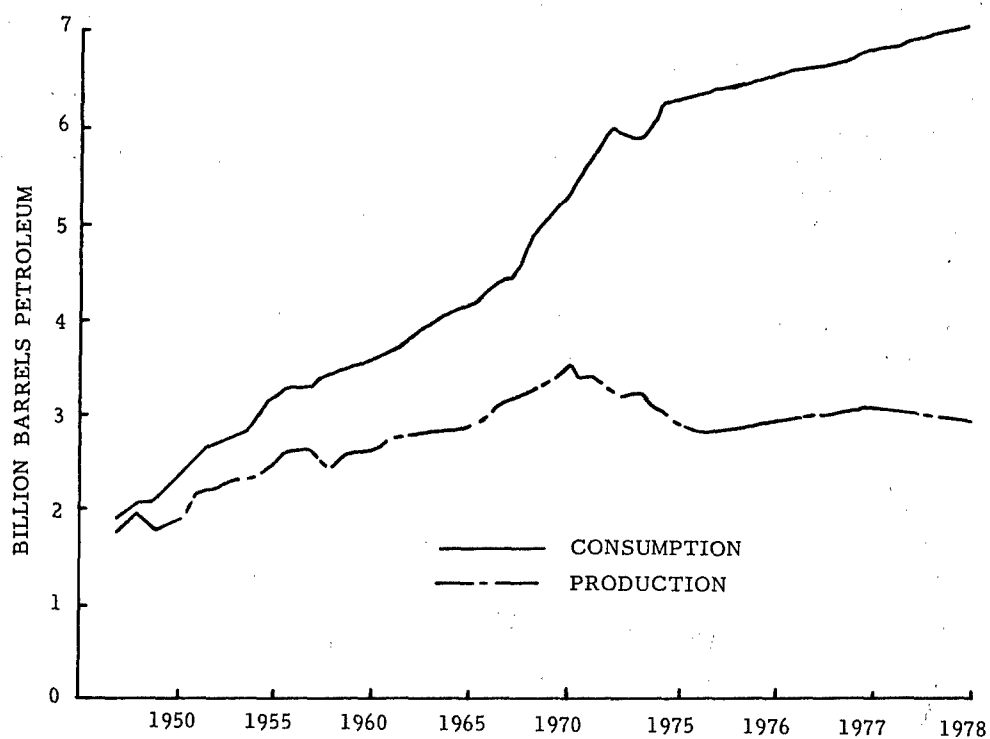


Figure 3. U. S. petroleum production and consumption 1947 - 1978.

TABLE 1. PROJECTION OF PRODUCTION OF COAL SYNFUELS  
DURING 1985 - 2000 (THOUSAND BBL PER DAY OIL EQUIVALENT)

Fuel Type	1985	1990	1995	2000
High Btu Gas	20-33.5	145-225	275-425	550-800
Low to Medium Btu Gas*	-	100-250	300-550	400-700
Liquids	-	125-175	475-600	675-1000
TOTAL	20-33.5	370-650	1050-1575	1625-2500

\*Predominantly Medium-Btu

Source: Hagler, Bailly & Company, based on capacity projections by DOE; Bechtel National, Inc.; Amoco; Booz-Allen; Council on Environmental Quality; Research Triangle Park

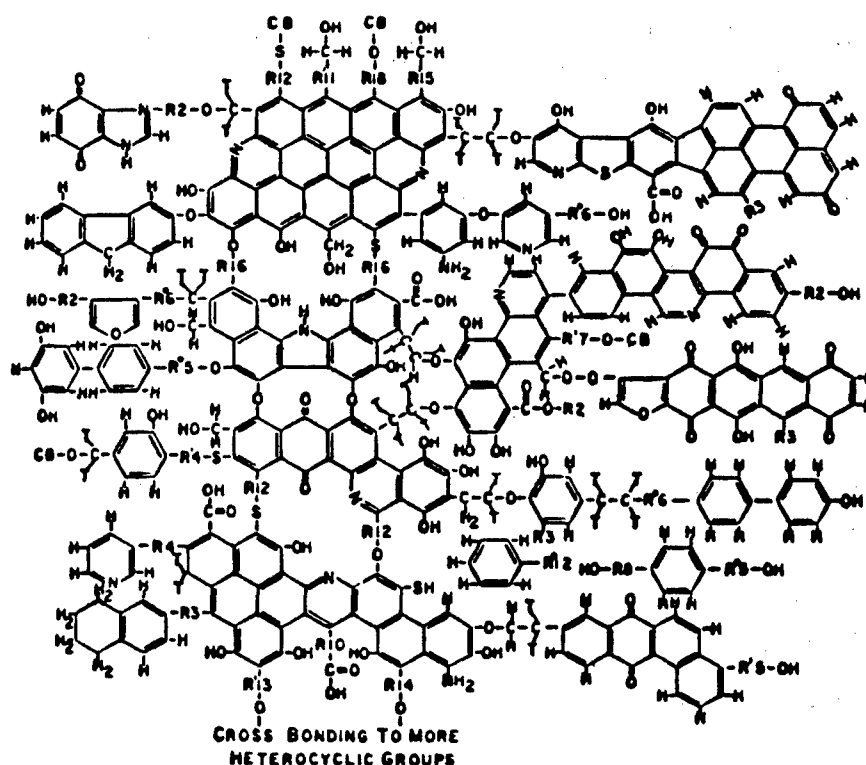
Coal conversion and oil shale retorting processes involve raw materials that contain many trace minerals hazardous to health. Also, they involve elevated temperatures to levels at which polynuclear aromatic hydrocarbons are produced. There is concern about potential health risks to both occupational workers and to the populations surrounding the synfuel plants. In fact, potential health impacts of the synfuel industry have been recognized as one of the important constraints on further developments of this industry (U. S. Department of Energy, 1979; Hill, 1979). Still, the state of knowledge about any quantification of the potential health risk has been summarized as follows: "The risk factors are still largely unidentified because knowledge is lacking about the kinds and quantities of toxic materials to be released from actual synfuel plants" (U. S. Department of Energy, 1979). A review of the literature pertaining to health risks follows, after a short review of the synfuel processes.

### SYNFUEL PROCESSES

#### GENERAL PROCESSES

The chemistry of coal has been extensively studied. The conventional view is that coal is basically constructed of aromatic/hydroaromatic structures as shown in Figure 4 (Fluor Engineers and Constructors, Inc., 1979a). Although the structure of coal is an important key to the understanding of what chemicals are produced during conversion, our knowledge of it is still lacking. Recent findings indicate new structures, including one acid-base structure similar to that found in asphaltenes (Fluor Engineers and Constructors, Inc., 1979a).

The structure of bituminous coal is better understood than that of other types of coal. It consists of clusters of aromatic carbon rings with appreciable amounts of hydroaromatic carbon. An average of 65 to 75% of the carbon is aromatic, 15 to 25% is hydroaromatic, and 5 to 10% is aliphatic.



$R^N$  = Alicyclic rings of N carbons.  
 $RN$  = Alkyl side chain of N carbons.  
 $R'N$  = Unsaturated alkyl side chain of N carbons.  
 $CB$  = Cross bonding by O or S to new heterocyclic groups with side chains.  
 $T$  = Tetrahedral 3 dimensional C-C bonds, C-O bonds and C-S bonds.

Source: U.S. Patent 3,244,615

Figure 4. Possible chemical structure for coal.

Coal conversion or oil shale retorting are defined as the upgrading of these materials to produce more marketable products. In the case of coal, the hydrogen to carbon ratio is increased to form liquid or gaseous hydrocarbons (Figure 5). In the case of oil shale, the oil-like kerogen in shale is extracted, to be possibly followed by further upgrading to petroleum like gasoline.

There are hundreds of processes for the conversion of coal or for the retorting (heating) of oil shale to gaseous, liquid, or solid end products. The more important ones can be categorized as follows:

- (1) Coal gasification to low, medium, or high Btu gas.
- (2) Coal liquefaction to hydrocarbon liquids.
- (3) Oil shale retorting to hydrocarbon liquids.

C/H  
Ratio

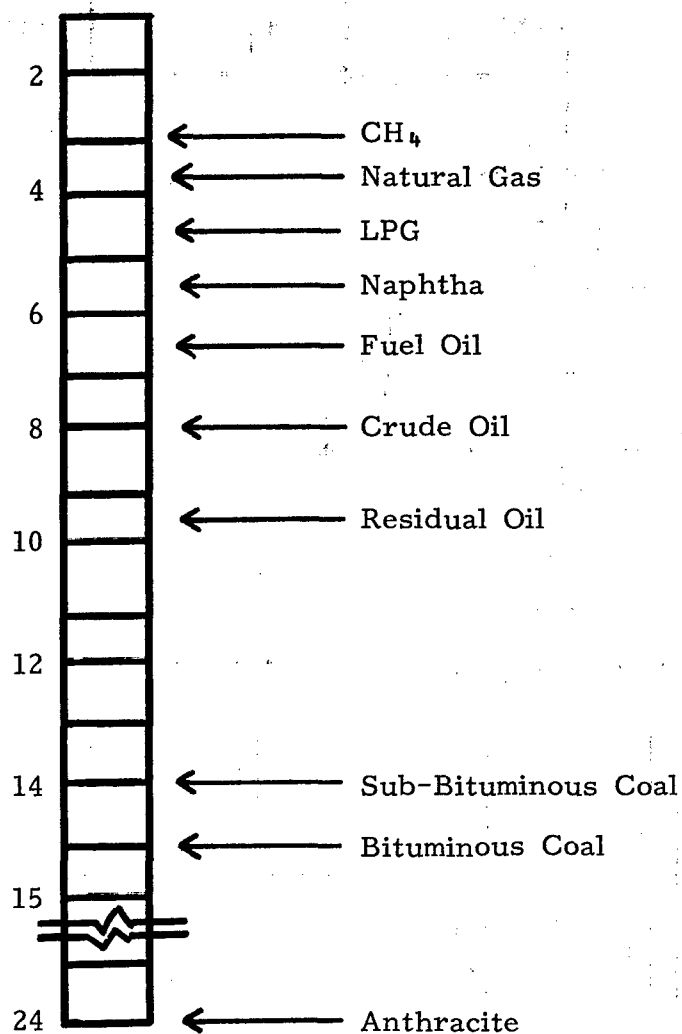


Figure 5. Carbon/Hydrogen ratio for gaseous, liquid, and solid fossil fuels.

Their schematic diagrams are given in Figure 6 (Fluor Engineers and Constructors, Inc., 1979b), Figure 7 (Fluor Engineers and Constructors, Inc., 1979a), Figure 8 (Fluor Engineers and Constructors, Inc., 1979c), and Figure 9 (Fluor Engineers and Constructors, Inc., 1979c).

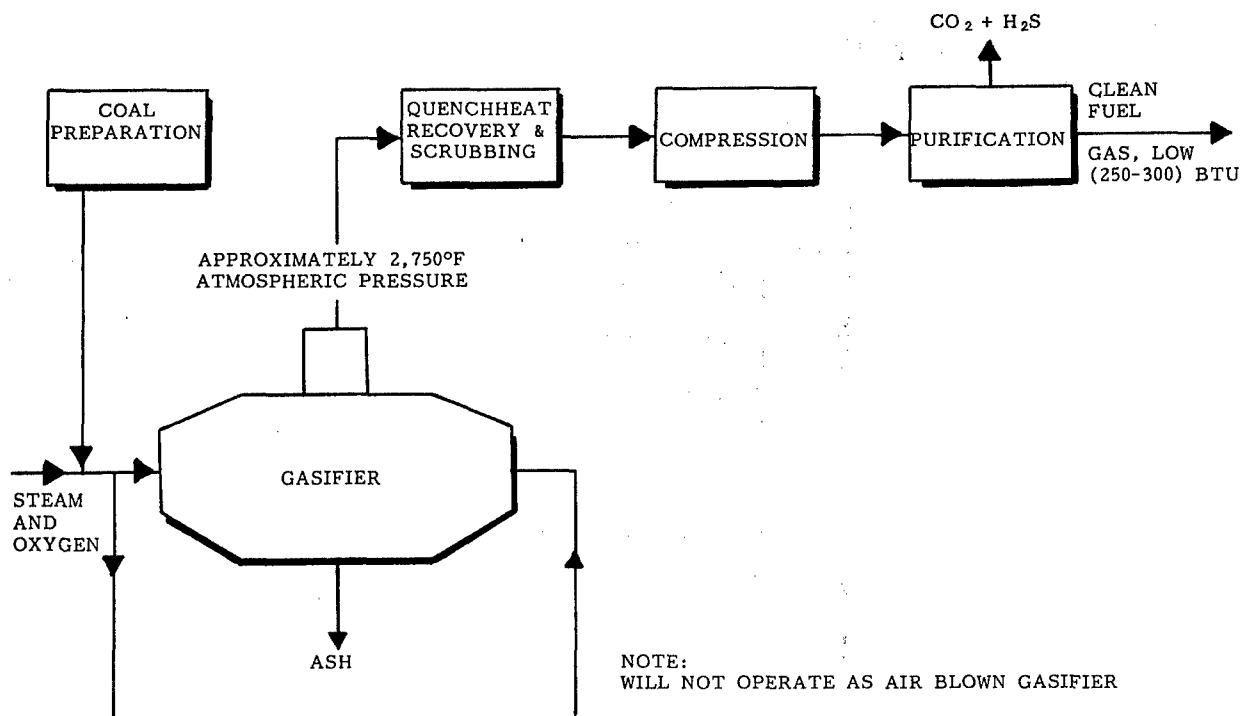


Figure 6. Simplified flowsheet diagram of coal gasification.

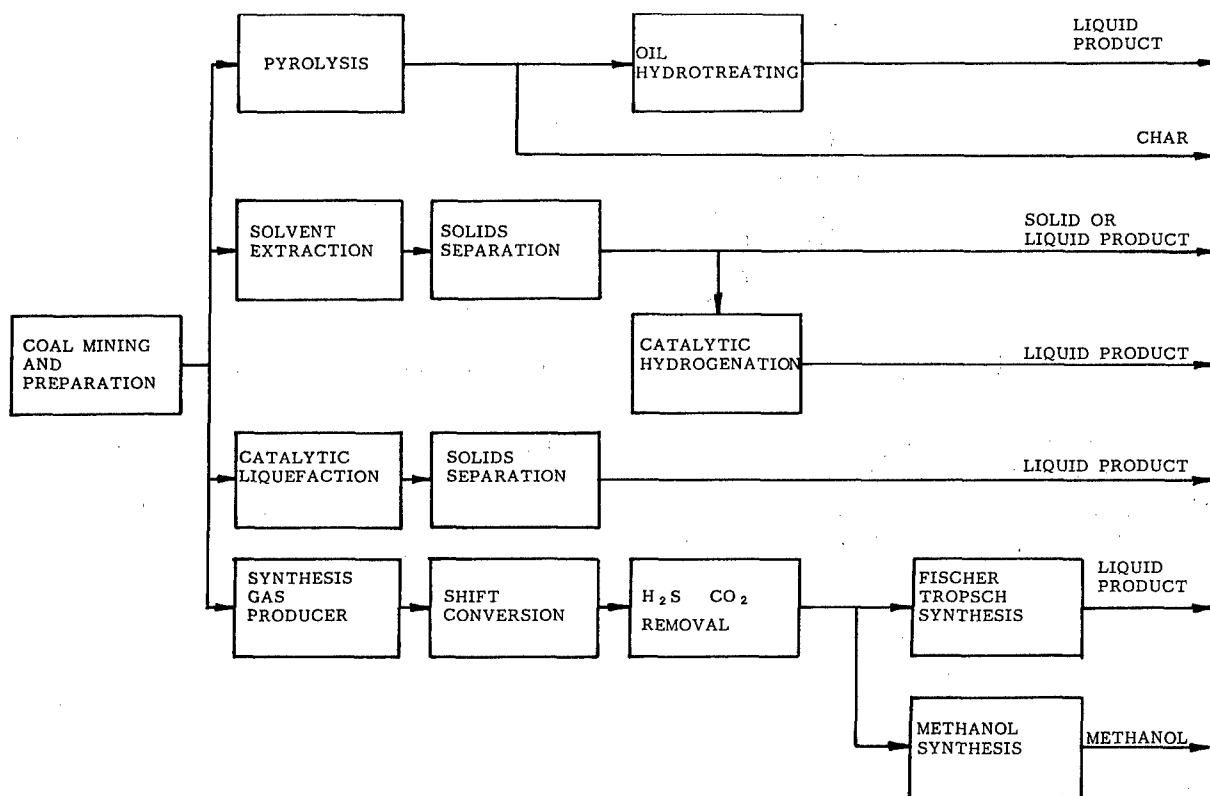


Figure 7. Coal liquefaction routes.

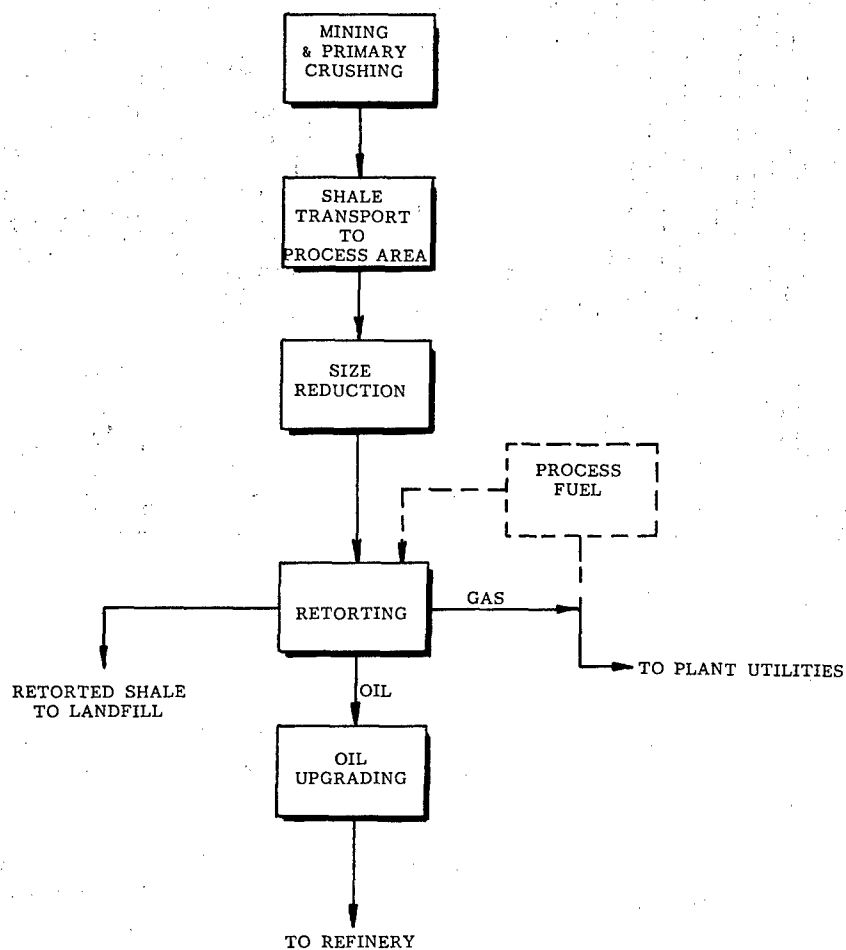


Figure 8. Generalized schematic flowsheet for surface processing of oil shale.

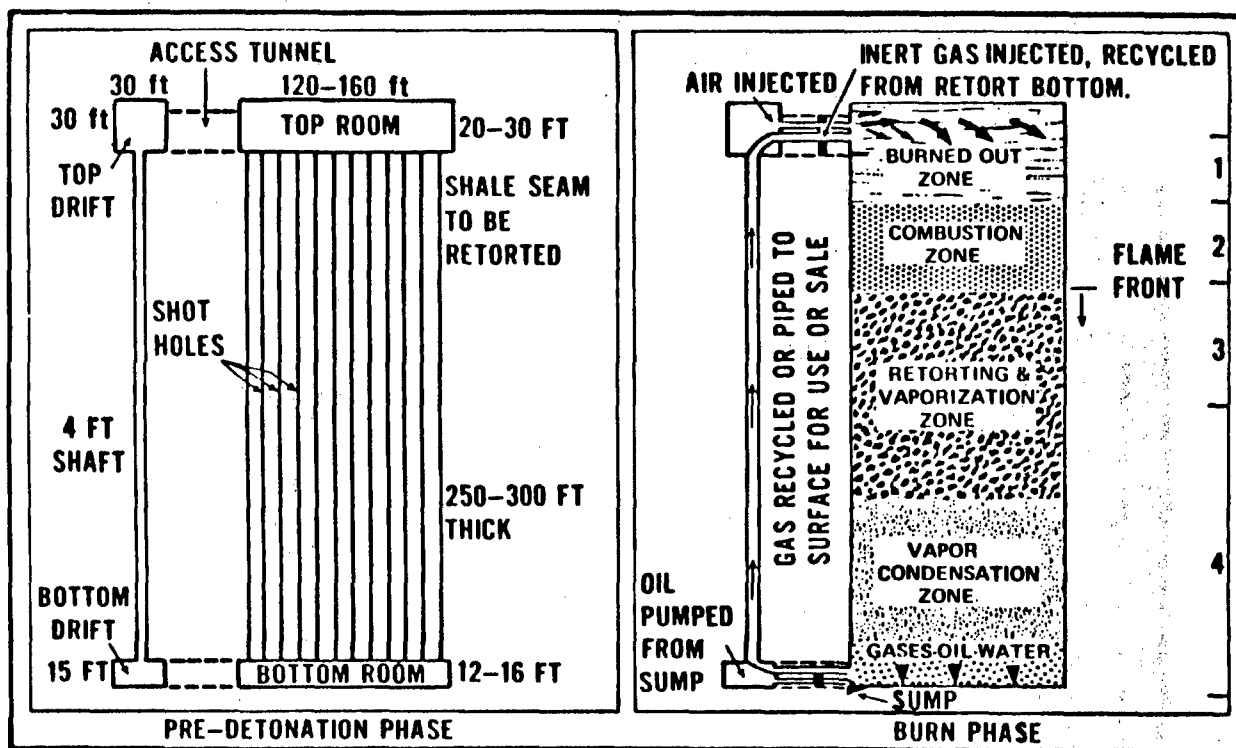


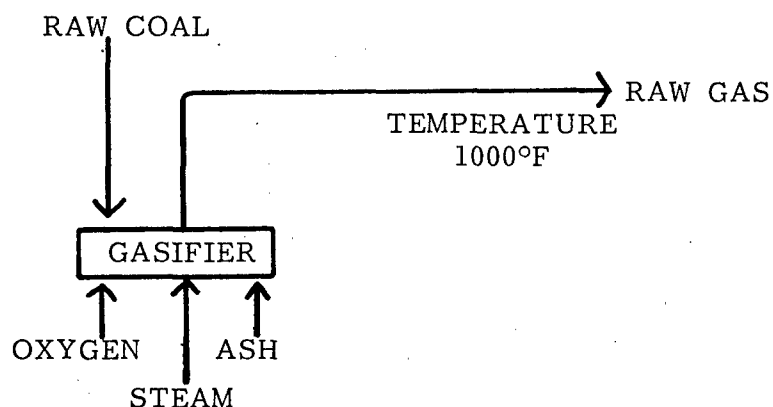
Figure 9. Schematic flowsheet of In Situ processing of oil shale.

## COAL GASIFICATION

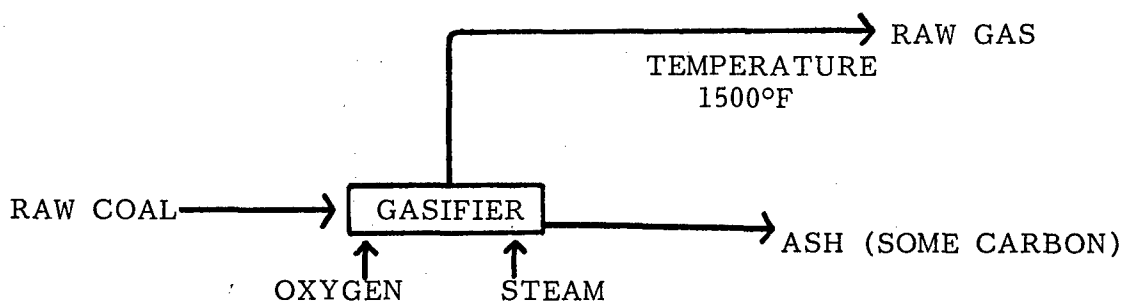
Coal gasification processes can be divided into three major process types according mainly to the way in which the feedstock coal, steam, and the product gases are contacted. They are:

1. Fixed bed gasification in which the crushed sized coal is fed from the top of the reactor vessel. Steam, air or oxygen is blown upwards.
2. Fluidized bed gasification in which the finely sized coal particles are "fluidized" by the steam, air or oxygen which is piped through them.
3. Entrained bed gasification in which the even finer coal particles are blown into the reacting gas stream prior to entry into the reactor. The coal particles are suspended in the gas phase, and are filtered and recycled into a product gas with a suitable heating value.

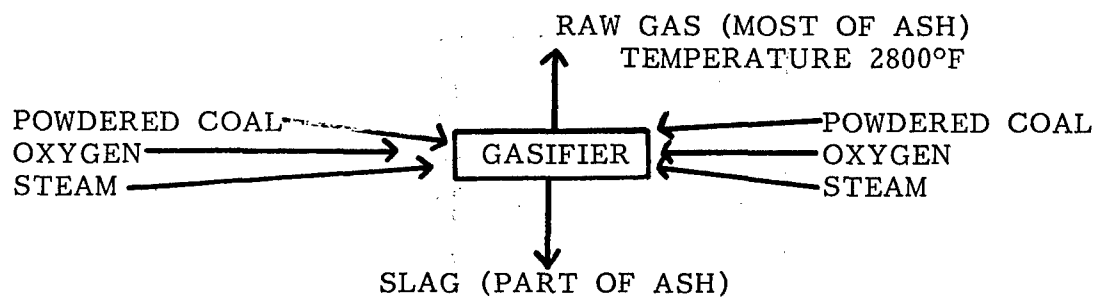
Figure 10 (Bentz and Associates, 1980) describes the main features of these three processes.



FIXED BED GASIFICATION e.g. LURGI



FLUID BED GASIFICATION e.g. WINKLER

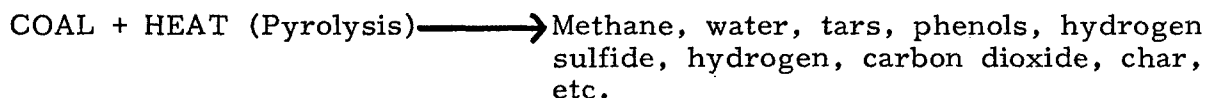


ENTRAINED GASIFICATION e.g. KOPPERS-TOTZEK

Figure 10. Basic coal gasification processes.



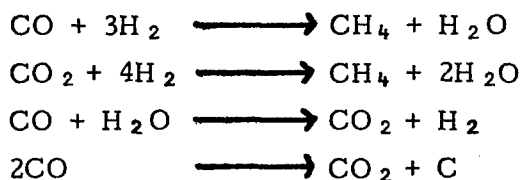
The heart of the gasification process is the devolatilization of the coal. It follows the following general reaction.



The pressures used for gasification range from atmospheric pressure to 1000 psi. The heat required to maintain the endothermic gasification reaction is supplied from burning coal. Air or oxygen are also needed to support the combustion reaction. If air is used, the product is low Btu gas ranging from essentially a carbon monoxide/hydrogen mixture (Koppers-Totzek process) to mixtures containing various proportions of carbon monoxide, carbon dioxide, hydrogen, water, methane, hydrogen sulfide, nitrogen, and typical products of pyrolysis such as tar, oils, phenols, etc. The product's heat value ranges between 100 to 300 Btu per standard cubic foot (Cameron Engineers, Inc., 1978). If oxygen is used, medium Btu gas results. Its heat value ranges between 300 and 700 Btu per scf (Cameron Engineers, Inc., 1978).

If the desired final product is either low- or medium-Btu gas, gasification is usually followed by scrubbing and cleaning. Carbon dioxide and sulfur compounds are partially removed, and the resulting gas is used directly. If high-Btu gas is desired, hydrogenation is required. High Btu gas has a heating value of approximately 1000 Btu per scf (Cameron Engineers, Inc., 1978). It is compatible with natural gas and is produced by hydrogenation over a catalyst such as iron, cobalt, nickel, ruthenium, rhodium, palladium, osmium, iridium, and platinum.

The following exothermic reactions occur simultaneously (Cameron Engineers, Inc., 1978):



Hydrogen for these reactions is produced from coal according to the reaction



### COAL LIQUEFACTION

There are two basic routes to coal liquefaction, direct and indirect liquefaction. In direct processes, slurried crushed coal is reacted directly with hydrogen at high temperature and pressure conditions to produce liquid hydrocarbons. In indirect liquefaction, coal is first gasified to produce a hydrogen and carbon monoxide mixture. Further recombination with the aid of a catalyst produces liquid products.

Direct liquefaction is further broken down into three generic processes: pyrolysis, solvent extraction, and catalytic liquefaction. In pyrolysis processes, coal is heated to temperatures above 750°F. It is converted into gases, liquids, and char. Solvent extraction makes use of coal that goes into solution. The "donor" solvents act as a source of hydrogen to the coal products and are reacted together at temperatures up to 950°F. In catalytic liquefaction, pulverized coal is mixed with 1-1.5 parts of recycle solvent. A suitable catalyst is used to add hydrogen. Most processes of this type operate in the liquid phase with catalyst dispersed throughout or in a fixed bed. Temperatures of about 950°F are used. Indirect liquefaction is typified by a two stage conversion of the coal or its product syngas to liquid hydrocarbons. Coal is first reacted with steam and oxygen to produce a gas composed primarily of carbon monoxide and hydrogen. This gas stream is subsequently purified to remove sulfur, nitrogen, and ash. The product gas is then catalytically reacted to yield hydrocarbon products.

### OIL SHALE RETORTING

There are two major routes for converting oil shale to liquid or gaseous fuels:

- 1) Conventional mining followed by surface retorting (heating), and
- 2) In situ retorting.

In addition, there is modified in situ. In this process, the permeability (i.e., void volume) of oil shale deposits is increased in order to enhance the in situ retorting by removing some of the shale.

In surface retorting, the crushed shale is heated in a vessel to between 800°F and 1000°F. Gas or noncombustible solids such as sand or ceramic balls can be used as heat carriers. The vapor produced during the heating is condensed to form crude shale oil. It can be further upgraded and refined to produce more marketable products.

In the in situ retorting of oil shale, explosives are used to create a rubble filled chamber. Other methods of rubblizing are being tried. The chamber functions as a batch retort with the application of an outside energy source to begin the retorting reaction. Usually, off-gas or oil from other oil shale retorts is used to begin the operation. It can then continue on its own. The flame front commenced by the external heating source moves downward. It is preceded by the liquid and gaseous products which are evolved from the shale by the retorting action of the hot, oxygen-deficient combustion gases. The liquids collect in a sump, from which they are pumped to the surface.

### UPGRADING OF LIQUID PRODUCTS

The problem of upgrading liquid products so that they can be suitable for substitution for petroleum crudes is mainly limited to fuels used for transportation.

Preliminary studies indicate that upgrading accomplished by an increase in H/C ratio and a reduction in the aromatics and organic nitrogen contents of synthetic crudes is feasible, although expensive in terms of costs and energy losses (U. S. Department of Energy, 1979; Sullivan et al., 1980; Gilder and Burton, 1980). This is primarily done by the hydrogenation of the crudes. Oil from shale with hydrogen to carbon ratio of 1.9 (vs 2 for petroleum) can be substituted for present fuels with relative ease. Oil from coal conversion with hydrogen to carbon ratio of 0.75 requires considerable upgrading (U. S. Department of Energy, 1979).

The hydrogenation process consists of heating the oil feed to 600°-850°F and flowing it through a catalyst in the presence of hydrogen at 600 to 3000 psi pressure.

### COMPARISON OF SYNFUELS PROCESSES IN RESPECT TO CONVENTIONAL POLLUTANTS

Eight coal conversion processes are compared to coal utilization for direct heating and for electricity production using best available control technologies (Salmon, 1980). Table 2 (Salmon, 1980) summarizes the processes under comparison. The impacts are evaluated within the framework of complete energy source to end use systems. Coal exploration and mining, transportation, production of fuel, and distribution to consumers have all been examined.

TABLE 2. DESCRIPTION OF COAL ENERGY SYSTEMS

Plant A	- is a $50 \times 10^3$ BPD coal liquefaction plant producing $45 \times 10^3$ bbl of oil daily, averaging $5.6 \times 10^6$ Btu/bbl, and utilized directly by end users.
Plant B	- is a $97 \times 10^3$ BPD coal liquefaction plant producing $88 \times 10^3$ bbl of oil daily, averaging $5.6 \times 10^6$ Btu/bbl, and utilized for generating electricity in a 2800-MWe power plant with FGD, which produces 2100 MWe of electricity for end users.
Plant C	- is a 2800-MWe electric power plant with FGD, producing 2100 MWe of electricity
Plant D	- is a 2800-MWe electric power plant with AFBC, producing 2100 MWe of electricity.
Plant E	- is an AFBC plant producing $180 \times 10^{12}$ Btu heat/year.
Plant F	- is a $300 \times 10^7$ s.c.f./day low-Btu coal gasification plant producing $270 \times 10^7$ s.c.f. of gas daily with 100 Btu s.c.f. gas utilized directly by end users.
Plant G	- is a $430 \times 10^7$ s.c.f. of low-Btu gas daily with gasification plant producing $430 \times 10^7$ s.c.f. of low-Btu gas daily with 100 Btu s.c.f., integrated with 2800 MWe combined cycle electric power plant with FGD producing 2100 MWe of electricity for end users.
Plant H	- is a $930 \times 10^6$ s.c.f./day medium-Btu coal gasification plant producing $830 \times 10^6$ s.c.f. of gas daily with 300 Btu s.c.f. for direct utilization by end users.
Plant I	- is a $1590 \times 10^6$ s.c.f./day medium-Btu coal gasification plant producing $1430 \times 10^6$ s.c.f. of gas daily with 300 Btu s.c.f., integrated with 2800 MWe combined cycle electric power plant with FGD producing 2100 MWe of electricity for end users.
Plant J	- is a $280 \times 10^6$ s.c.f./daily high-Btu coal gasification plant producing $250 \times 10^6$ s.c.f. of gas daily with 1000 Btu s.c.f. utilized directly by end users.
Plant K	- is a $480 \times 10^6$ s.c.f./day high-Btu coal gasification plant producing $430 \times 10^6$ s.c.f. of 1000 Btu s.c.f. gas daily, integrated with 2800 MWe combined cycle electric power plant producing 2100 MWe of electricity for end users.

## Source to End Use Systems Examined

End Use	Conversion to Fuel for Direct Use	Conversion to Electricity
Residential Heating	High-Btu gas	Low-Btu gas (combined cycle), conventional combustion, direct (fluidized bed) combustion, coal liquids (e.g., SRC)
Commerical and Industrial or Space Heating	High-Btu gas, Low-Btu gas, FBC	Low-Btu gas, conventional combustion, FBC
Direct Power Generation		Low-Btu gas, conventional combustion, FBC, coal liquids (e.g., SRC)

The sizes of the various coal systems evaluated in this reference are so chosen that each produces an equivalent quantity of useful energy. Thus, the system factors, indices of potential impacts, and estimated impacts are quantified for equal amounts of useful energy. They represent the potential benefits from the energy systems. A model plant producing  $63 \times 10^{12}$  Btu/year of useful energy at the point of end use is the standard reference. It is equivalent to a gasification plant producing daily 250 million cubic feet of high Btu gas.

Table 3 (Salmon, 1980) summarizes the indices of major potential health, safety, and environmental impacts from unit energy systems of the various coal energy systems of Plants A through K described previously. A conventional oil energy system (Plant L) is also included in this table for comparison with the coal systems. The following indices are employed:

- Coal consumption provides an index of the major occupational health impacts to miners from respiratory and mining accident impacts and is also an index of general public coal transportation impacts.
- SO<sub>2</sub> emissions provide an index of the respiratory health impacts to the general public from combustion products, of the damages to plants, animals, soils, and ecosystems from combustion products, and of the damages to property and to agricultural production.
- CO<sub>2</sub> emissions provide an index of long-term weather modifications.
- Land use is indicative of land impacts, mainly to plant and animal species and to ecosystems, and of socioeconomic impacts.
- Water consumption and manpower requirements are related to socioeconomic impacts.

TABLE 3. INDEXES OF MAJOR POTENTIAL HEALTH, SAFETY, AND ENVIRONMENTAL IMPACTS FROM UNIT ENERGY SYSTEMS PRODUCING ANNUALLY  $63 \times 10^{12}$  BTU OF USEFUL ENERGY

Index	Related Impact	A	B
		Coal Combustion	Liquefaction Electricity
Annual Quantities Mined Coal ( $10^6$ tons/year)	Occupational mining impact (OWP and mining accidents)  General public impact from transportation	6.6	13
Annual emissions of $\text{SO}_2$ ( $10^3$ tons/year)	General public impact from combustion products  Damages to environment  Damages to property and agricultural products	13	29
Annual emissions of* $\text{CO}_2$ ( $10^6$ tons/year)	Long-term weather modification	9.4	19
Land use ( $10^3$ acres/ per year)	Damages to land  Socioeconomic impact competing land uses	45	87
Peak Construction Manpower (men)	Socioeconomic impact community growth	2,600	4,000
Operational Manpower (men)	Socioeconomic impact community growth	2,000	2,900
Water consumption ( $10^9$ gallons/year)	Socioeconomic impact competing water uses	1.1	14

\* These figures do not include  $\text{CO}_2$  production from  $\text{H}_2$  generation for gasification or liquefaction processes. An additional amount of  $\text{CO}_2$  (approximately 35% by weight) derives from these sources.

TABLE 3. CONTINUED

## Power Plants Compared

C Electrical Power with FGD	D Electrical Power with AFBC	E AFBC Combustion	F Low-Btu Gasification Combustion	G Gasification Electricity
8.6	8.1	10.0	5.9	9.4
33	35	43	9.5	15
20	19	24	7.5	12
62	61	68	40	64
2,900	2,900	3,500	1,800	3,100
1,300	1,300	1,500	940	1,500
12	7.1	10	1.0	10

TABLE 3. INDEXES OF MAJOR POTENTIAL HEALTH, SAFETY, AND ENVIRONMENTAL IMPACTS FROM UNIT ENERGY SYSTEMS PRODUCING ANNUALLY  $63 \times 10^{12}$  BTU OF USEFUL ENERGY (CONTINUED)

Index	Related Impact	H Medium-Btu Combustion	I Gasification Electricity
Annual Quantities Mined Coal ( $10^6$ tons/year)	Occupational mining impact (OWP and mining accidents)  General public impact from transportation	6.2	11
Annual emissions of SO <sub>2</sub> ( $10^3$ tons/year)	General public impact from combustible products  Damages to environment  Damages to property and agricultural products	8.7	17
Annual emissions of CO <sub>2</sub> ( $10^6$ tons/year)	Long-term weather modification	7.1	12
Land use ( $10^3$ acres/ per year)	Damages to land  Socioeconomic impact competing land uses	42	72
Peak Construction Manpower (men)	Socioeconomic impact community growth	3,500	4,900
Operational Manpower (men)	Socioeconomic impact community growth	1,100	1,700
Water consumption ( $10^9$ gallons/year)	Socioeconomic impact competing water uses	1.2	12

TABLE 3. CONTINUED

Power Plants Compared		
J High-Btu Combustion	K Gasification Electricity	L Conventional Oil
7.2	13	N. A.
8.2	19	12
7.4	13	7.8
49	84	-----
5,200	5,500	-----
1,400	2,100	0
1.7	14	16



TABLE 4. MAJOR ANNUAL HEALTH AND SAFETY IMPACTS FROM SOME ENERGY SYSTEMS BASED ON UNIT PLANTS (CASES/YEAR)

Impacts	Coal Liquefaction		Electrical Power with		AFBC
	<u>A</u> Combustion	<u>B</u> Electricity	<u>C</u> FGD	<u>D</u> AFBC	<u>E</u> Combustion
<u>OCCUPATIONAL HEALTH IMPACTS</u>					
Disease deaths from extraction <sup>a,b</sup>	1.7 - 7.8	3.4 - 15	2.3 - 10	2.1 - 9.6	2.6 - 12
Damage to health from extraction <sup>c</sup>	67 - 210	130 - 410	87 - 270	82 - 250	100 - 330
Accidental deaths from extraction <sup>d</sup>	0.83 - 1.4	1.6 - 2.8	1.1 - 1.9	1.0 - 1.8	1.3 - 2.2
Accidental injuries from extraction <sup>d</sup>	49 - 100	97 - 200	64 - 130	60 - 120	74 - 150
Accidental deaths from transportation <sup>e,f</sup>	0.56	1.1	0.72	0.68	0.84
Accidental injuries from transportation <sup>e,f</sup>	7.2	14	9.2	8.7	11
Accidental deaths from conversion <sup>g,h</sup>	0.36	0.62	0.33	0.33	0.37
Accidental injuries from conversion <sup>g,h</sup>	11	19	13	13	16
Disease deaths from conversion <sup>j</sup>	0.26	0.30	0	0	0
<u>GENERAL PUBLIC HEALTH IMPACTS</u>					
Accidental deaths from transportation <sup>e,f</sup>	5.0	9.9	6.5	6.1	7.6
Accidental injuries from transportation <sup>e,f</sup>	6.5	130	84	79	98
Disease deaths from emissions <sup>k,m</sup>	0 - 8.8	0 - 20	0 - 22	0 - 24	0 - 29
Aggravation of asthma from emissions <sup>m,n</sup>	0 - 13,000	0 - 28,000	0 - 32,000	0 - 34,000	0 - 42,000

TABLE 4. CONTINUED

PROCESS COMPARED						
<u>Low-Btu Gasification</u>		<u>Medium-Btu Gasification</u>		<u>High-Btu Gasification</u>		<u>Conventional</u>
<u>F</u>	<u>G</u>	<u>H</u>	<u>I</u>	<u>J</u>	<u>K</u>	<u>L</u>
Combustion	Electricity	Combustion	Electricity	Combustion	Electricity	Electricity Oil
1.6 - 7.0	2.5 - 11	1.6 - 7.3	2.8 - 13	1.9 - 8.3	3.3 - 16	0
60 - 190	95 - 300	63 - 190	110 - 330	73 - 230	130 - 390	0
0.74 - 1.3	1.2 - 2.0	0.78 - 1.3	1.3 - 2.3	0.91 - 1.6	1.7 - 2.7	---
44 - 90	70 - 140	46 - 95	79 - 160	54 - 110	93 - 180	0.0063 - 0.0070
0.50	0.79	0.52	0.89	0.61	1.1	0.042
6.4	10	6.7	11	7.8	14	0.73
0.47	0.79	0.58	0.96	0.74	1.1	0.04 <sup>L</sup>
16	28	21	34	27	43	3.5 <sup>L</sup>
0.05	0.08	0.07	0.10	0.09	0.12	0.01
4.5	7.2	4.7	8.1	5.5	9.5	0 <sup>L</sup>
58	92	61	100	71	120	0 <sup>L</sup>
0 - 6.5	0 - 10	0 - 5.9	0 - 12	0 - 3.8	0 - 13	0 - 8.2
0 - 9,300	0 - 15,000	0 - 8,500	0 - 17,000	0 - 8,000	0 - 19,000	0 - 12,000

Table 4 (Salmon, 1980) summarizes the major annual health and safety impacts from the various energy systems. In deriving the estimates of health impacts from air pollutants the following ranges were used (Ramsay, 1978):

- 0 to 0.68 deaths per 1000 tons of SO<sub>2</sub> emissions, and
- 0 to 980 cases of aggravation of asthma per 1000 tons of SO<sub>2</sub> emissions.

The lower value of zero is chosen to reflect the large uncertainties involved in the assessments and to indicate the considerable judgmental value involved.

The following conclusions are drawn from Table 4:

- The occupational health impacts associated with the mining of coal are the predominant ones, and mask all others. Because mining impacts depend mainly on the overall energy efficiencies of the coal systems, the smallest impacts of conversion technologies are associated with the direct use of low-Btu gasification (overall efficiency is 49 percent). The largest impacts are associated with the use of liquid products from coal conversion for electricity generation (efficiency is 22 percent).
- Coal conversion technologies that convert the fuels to electricity have larger impacts than the corresponding plants with direct fuel uses. The occupational health impacts from the latter type of plants are about twice as large as those from corresponding plants because of greater consumption of coal and greater emissions of pollutants. Conversion technologies that involve direct utilization of the fuels produced by them manifest smaller occupational health impacts than electrical power plants.
- The key public health impacts from the various coal systems are those from emissions of combustion products and from coal transportation. The health impacts to the general public from coal conversion systems are considerably smaller than from an equivalent coal-electric system, the main reason being that most of the health impacts from environmental pollutants are associated with combustion products. Only about 10 percent of the coal undergoes combustion in conversion processes, and the synthetic fuels produced are cleaned of sulfur prior to utilization. For coal gasification, the sulfur is removed almost entirely in conversion and the later combustion of the gas emits very little sulfur. For coal liquefaction, some sulfur still remains in the coal liquids and is later emitted upon combustion.
- Thus, considerably smaller releases of SO<sub>2</sub> are emitted from coal conversion systems than from coal-electricity. This advantage is partly offset by the fact that synthetic oil and gas are burned closer to the public than coal in electric power plants. But there is not an equivalent amount of pollutant emissions released. Accidental deaths and injuries to the general public are also smaller for coal conversion systems because of their higher overall energy efficiencies.

## IMPACTS FROM TRACE CONTAMINANTS

Coal and oil shale contain a large number of trace elements. The ones of concern include arsenic, cadmium, chromium, lead, mercury, nickel, the radio-nuclides uranium and thorium, and various associated radioactive decay products. These contaminants can reach the environment either before, during, or after combustion or conversion. The routes of entry of the contaminants into the human body are through inhalation of contaminated air or through ingestion of contaminated water or food. The main sources of human exposure include runoff and seepage from mining, storage, and waste piles, air emissions from fires of coal and coal waste storage piles, the conversion of coal and oil shale, the upgrading of the products, and their utilization.

There is a concern about the possible significance to human health of those trace elements that become mobilized during the coal conversion and oil shale retorting fuel cycles. Many of them are known to be highly toxic at high doses (U. S. Research and Energy Development, 1977). They can cause damage directly through toxic metabolic interactions or by catalyzing the effects of other pollutants (Amdur, 1976; Nordberg, 1974). The response is further complicated by individual nutritional and immunologic status (Nordberg, 1974). However, the evaluation of the effects of low doses is obscured by the fact that the exposures are usually to multiple pollutants. The extent to which bioconcentration mechanisms in the environment affect the doses causes further obscurities. In fact, it becomes difficult to draw any conclusions about the hazards to man.

The conclusions of a National Academy of Sciences Panel on this subject (National Academy of Sciences, 1980) were "despite the voluminous biochemical and toxicologic literature on trace-metal effects, the nature and size of the human response to the chronic, extremely low levels of expected metal exposures from coal-fuel-cycle processes is, as yet, poorly understood, as are the characterizations of emissions from many processes". Since toxicity of trace elements is often related to physiochemical states, detailed emissions characterizations are an important facet of health effects assessment (National Academy of Sciences, 1980).

Factors that could affect the routine release of trace elements to the environment may be categorized as coal-dust emissions, flue-gas emissions, and water contamination (from ash leachate and process condensate).

The effects of exposure of miners to the intake of trace elements in blown dust have not been studied directly. However, the U. S. Public Health Service studied the prevalence of Coal Workers Pneumoconiosis (CWP) and its relationship to dust content including trace elements. CWP was shown not to be significant in surface mines (Fairman et al., 1977). However, certain trace elements could act as factors or cofactors in CWP found among underground miners. No correlation could be found between the severity of CWP and concentrations of chromium, copper, manganese, nickel, titanium and zinc found in the lungs of deceased miners. However, vanadium, and to a lesser extent beryllium or magnesium concentrations, were found to be correlated to the severity of CWP (Sweet et al., 1978). This correlation by itself, however, is not proof of an effect. The increased retention of vanadium can be the

result of the disease rather than a cofactor causing it (Davis et al., 1977).

The predominant radionuclide exposures of coal miners are to radon and its daughters. A survey of the presence of radon and its daughters in the range of 0 to 0.3 working levels in 223 operating mines in 15 states concluded that there was no apparent occupational health hazard from inhalation of the ambient levels of these contaminants (Rock et al., 1975).

Exposure of the public to trace elements from surface mining can result from diesel fuel combustion and from coal fires. The regional emissions are low, ranging from  $10^{-2}$  ton/year to  $10^{-6}$  ton/year for various metals (National Academy of Sciences, 1980).

Radioactivity in coal mine drainage is predominantly from U-234, U-238, and their daughters. Some measurements (Caldwell et al., 1970) indicate low levels of up to 180 pCi/liter, but comprehensive studies have not been undertaken (National Academy of Sciences, 1980).

Trace elements are emitted during coal transport by trains. They are derived from diesel fuel combustion and from windblown coal particles. Occupational or public health impacts of trace metals from transport are not known. The emissions from low-level sources will be mobilized over shorter distances, with a risk of increased exposure for only relatively small populations. Regional emissions range from 10 ton/year to  $10^{-3}$  ton/year for various metals (National Academy of Sciences, 1980).

About 23 percent by weight of the mined coal ends as solid wastes from coal cleaning and processing. Good practice is to dispose of these wastes, whenever possible, by returning them to the mine. However, there are 3000 to 5000 abandoned coal waste piles in the United States (National Academy of Sciences, 1980). Their runoffs contaminate water supplies. It has been concluded (Wewerka et al., 1976) that trace metals mobilized in air and water from effluents of coal preparation constitute potential public health problems. However, the direct impact on public health is not known (National Academy of Sciences, 1980).

In the late 1960's, an estimated 300 to 500 coal-waste piles were burning as a result of intentional ignition or by spontaneous heat generated by reactions within the pile (National Academy of Sciences, 1980). Air pollution from burning piles transports the trace elements. The public health impact is not known (National Academy of Sciences, 1980).

Fly ash from coal combustion is used as a physical conditioner of waste sludges. No health effect has been documented (National Academy of Sciences, 1980).

Health effects from occupational exposure to trace metals from steam-electric plant processes have not been documented (National Academy of Sciences, 1980). These trace elements will be emitted as stack emissions. The toxic elements involved include antimony, selenium, nickel, vanadium, zinc, cobalt, bromine, manganese, and  $\text{SO}_4$ . They predominate in small particles emitted from high-temperature combustion sources (National Academy of Sciences, 1980). Table

5 (National Academy of Sciences, 1980) is a summary of the total amounts involved; little, however, is known about their fate. Sampling around power plants can account for less than 10 percent of the emissions within 50 miles of the plant. Their predicted levels in air are (except for beryllium) orders of magnitude less than acceptable concentrations (Crockett and Kinnison, 1977; Swaine, 1977; Vaughan et al., 1975). The predicted concentrations are given in Table 6 (U. S. Research and Development, 1977). A study of environmental transfer of trace elements from electric power plants (Vaughan et al., 1975) concluded that the ingestion of plants for food appears to be the only consequential pathway. Even this pathway does not become consequential until emissions become considerably greater (by perhaps orders of magnitude) than at present (National Academy of Sciences, 1980).

TABLE 5. AMOUNT OF TRACE METALS MOBILIZED INTO AIR BY STEAM-ELECTRIC PLANT COAL COMBUSTION AND BY COAL SUPPLY PROCESSES<sup>a</sup> BY U.S. REGION AND METAL, 1975 (U.S. TONS)<sup>b</sup>

Metal	Appalachia		Central		Northwest		Southwest	
	Supply	Combustion	Supply	Combustion	Supply	Combustion	Supply	Combustion
Arsenic	0.9	1	0.5	0.7	0.03	0.007	0.01	0.006
Beryllium	0.2	4	0.07	3	0.003	0.03	0.0002	0.1
Cadmium	0.03	—	0.04	100	0.001	0.02	0.0003	0.002
Chromium	0.9	100	0.6	200	0.09	7	0.01	3
Copper	1	—	0.5	10	0.2	1	0.01	0.6
Iron	0.9	10,000	0.0003	10,000	0.00007	20	0.000003	200
Lead	0.6	80	0.8	400	0.06	1	0.008	5
Mercury	0.01	40	0.009	20	0.001	80	0.01	0.3
Nickel	1	500	0.2	400	0.05	2	0.01	2
Vanadium	2	100	1	70	0.3	300	0.02	3
Zinc	2	100	10	700	0.08	60	0.02	7

<sup>a</sup>Includes extraction (underground, strip, and auger) and transportation (unit train, barge, truck). Air emissions from preparation were reported as being zero.

<sup>b</sup>Source: After Hittman Associates, Inc. (1977), 12 unpublished appendixes (numbered 4-15), on file in GERHD office, NRC.

TABLE 6. PREDICTED AIR CONCENTRATIONS OF AIRBORNE TRACE ELEMENTS IN THE VICINITY OF A 1400-MWe COAL-FIRED POWER PLANT COMPARED WITH ACCEPTABLE AIR CONCENTRATIONS<sup>a</sup>

Element	Predicted Air Concentrations ( $\mu\text{g m}^{-3}$ )	Acceptable Air Concentrations ( $\mu\text{g m}^{-3}$ )
As (V) or total	$1.2 \times 10^{-4}$	0.1
As (III)	—	$1 \times 10^{-5}$
Be	$2.9 \times 10^{-4}$	$5 \times 10^{-3}$
Cd	$1.2 \times 10^{-4}$	0.05
Cr	$7.2 \times 10^{-6}$	0.05
Cr (VI insol)	—	$1 \times 10^{-6}$
F	$2 \times 10^{-4}$	0.01
Hg	$1.2 \times 10^{-3}$	0.1
Hg organic	—	0.01
Mo	$2.4 \times 10^{-4}$	0.1
Ni	$4.8 \times 10^{-4}$	0.01
Ni carbonyl	—	$1 \times 10^{-6}$
Pb	$2.4 \times 10^{-4}$	1.0
Sb	$2.4 \times 10^{-5}$	0.1
Se	$2.4 \times 10^{-5}$	0.1
Ti	$3.0 \times 10^{-6}$	0.01
U	$3.0 \times 10^{-3}$	0.01
V	$2.4 \times 10^{-4}$	0.05

<sup>a</sup>Source: Van Hook and Shults (1976).

Radiation exposure from releases of radioactivity from power plants is estimated to be  $7 \times 10^{-3}$  mrem/yr per 1400-MWe coal fired plant (National Academy of Sciences, 1980). This is an inconsequential number relative to natural background of about 100 mrem/yr.

Acidification of fly ash waste can release trace elements to the environment. Also, they can be released from ponding and burial of sludge and slag. Information on this subject is very scant. One study (Fling, 1976) showed no concentrations of trace contaminants in ground water due to waste disposal operations. However, it is difficult to extrapolate these findings to other locations and soils because of the wide variability in soils for filtration. Measures to prevent contamination of groundwater and surface waters may be required (Bern, 1976).

In the case of coal conversion, oil upgrading and end product combustion processes, some of the trace elements can be volatilized. They may be released through leaks in the vessels or ovens or during combustion processes. Figure 11 (National Academy of Sciences, 1980) illustrates the effluent streams from coal conversion operations which can give rise to environmental releases. However, the degree of hazard, if any, involved in the releases depends on the trace element content of the feed coal, the specific processing conditions (particularly temperature and pressure which affect the conversion of trace elements to labile forms) and the control measures employed to reduce emissions.

The most troublesome unit operations have been discussed in the literature (National Academy of Sciences, 1980). They include:

- (1) Quenching and disposal of gasifier ash.
- (2) Gas scrubber water and condensate.
- (3) Acid gas removal system.
- (4) Tail gas from sulfur plant.
- (5) Catalyst disposal.
- (6) Product liquid utilization.

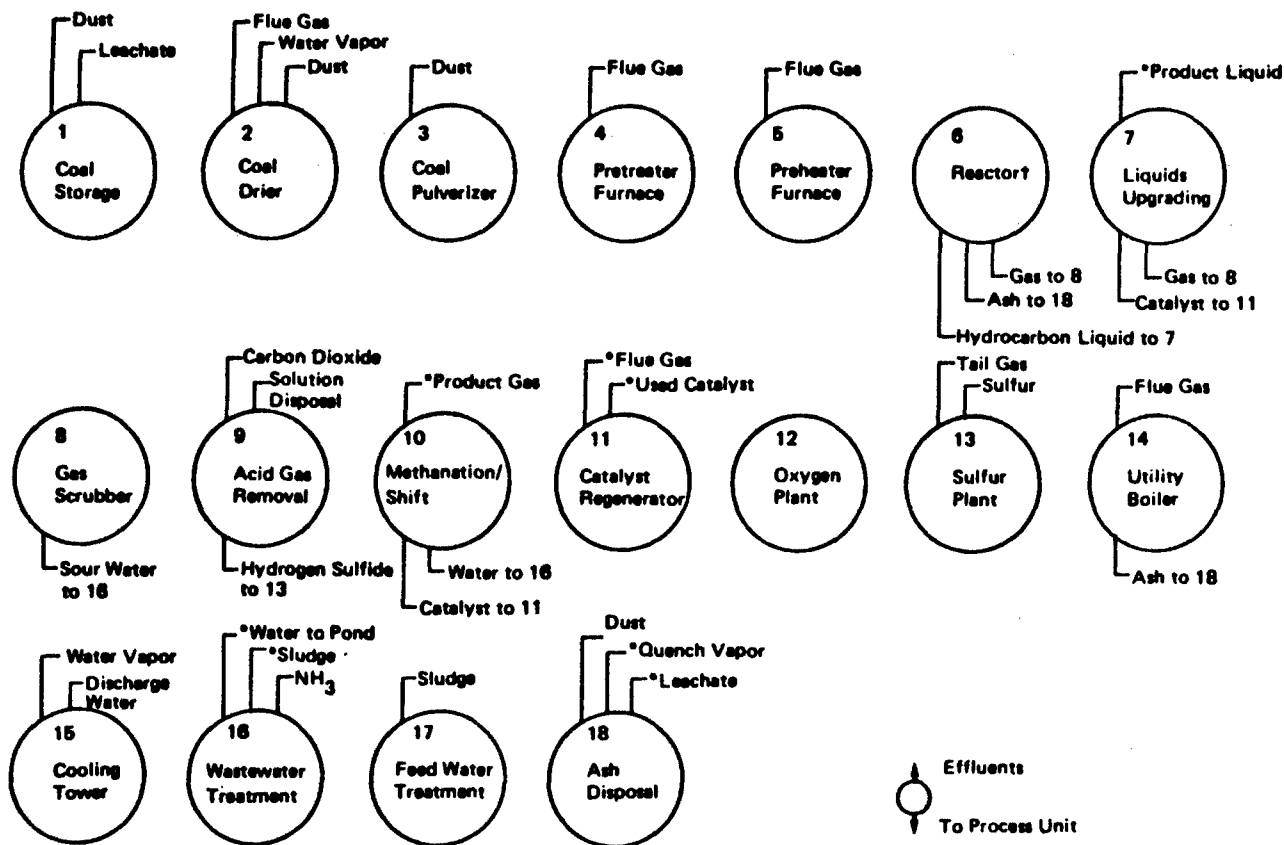
Product upgrading should be added to this list.

Tables 7 - 10 (National Academy of Sciences, 1980) summarize the known references about concentration of trace elements in various synfuel processes. However, there is no available information on the fate of trace elements during synfuel processes (National Academy of Sciences, 1980). There are only general discussions of the subject. Few generalizations can be drawn from the published literature. It is critical to know how selected trace elements are likely to partition into specific streams in any process, and between products, residues and effluents. Also important to know are the effects of process variables on the partitioning of elements; methods for treating effluents, particularly condensate and scrubber streams; and the leaching of trace elements from disposal sites.

In summary, there is insufficient knowledge to evaluate the potential health impacts from trace contaminants in coal synthetic fuel process.

In the case of oil shale retorting, our information is even more limited than for coal conversion. The basic information available in the literature is on the trace element content of raw shale oils, spent shale, and various process streams. Tables 11 - 14 (Baughman, 1978) and 15 (U. S. Department of Commerce, 1979) illustrate these concentrations.





\*Streams likely to be unique to synfuels processes compared to similar petroleum operations.

†Reactor (Item 6) may be liquefaction reactor, gasification reactor, or pyrolysis (coking) reactor and associated separations equipment.

Figure 11. Unit operations in liquefaction and gasification plants.

TABLE 7. SYNFUELS TRACE-ELEMENT CONCENTRATIONS IN SOLVENT REFINED COAL,  
SYNTHOIL, AND COAL-DERIVED OILS

Element	Process: Stream: Coal: Concentration: Reference:		Solvent Refined Coal (SRC)		SRC		Product SRC		Synthoil		Product Oil		Unspecified Bench-Scale Process	
			Product SRC Pittsburgh 8 ppm	Coleman et al. (1976)	Product SRC Unspecified ppm	Jahnig and Magee (1975)	Product Oil Western Kentucky Homestead ppm	Schultz et al. (1977)	Product Oil Utah ppm	Illinois 6	Kentucky	Given et al. (1975)		
Antimony	—	—	—	—	0.3	—	—	—	—	—	—	—	—	—
Arsenic	—	—	—	—	0.5	—	—	—	—	—	—	—	—	—
Beryllium	—	—	—	—	0.4	—	—	—	—	—	—	—	—	—
Boron	—	—	—	—	51	—	—	—	—	—	—	—	—	—
Cadmium	—	—	—	—	<0.1	—	—	—	—	—	—	—	—	—
Chromium	—	—	—	—	0.9	—	—	—	—	—	—	—	—	—
Copper	—	—	—	—	2.5	—	—	—	—	—	—	—	—	—
Iron	—	—	—	—	161	—	—	—	—	—	—	—	—	—
Lead	—	—	—	—	0.4	—	—	—	—	—	—	—	—	—
Manganese	—	—	—	—	1	—	—	—	—	—	—	—	—	—
Mercury	—	—	—	—	0.01	—	—	—	—	—	—	—	—	—
Nickel	—	—	—	—	4	—	—	—	—	—	—	—	—	—
Selenium	—	—	—	—	<1	—	—	—	—	—	—	—	—	—
Tin	—	—	—	—	9	—	—	—	—	—	—	—	—	—
Vanadium	—	—	—	—	16	—	—	—	—	—	—	—	—	—
Zinc	—	—	—	—	3	—	—	—	—	—	—	—	—	—

TABLE 8. SYNFUELS TRACE-ELEMENT CONCENTRATIONS AND PERCENTAGE OF  
FEED ELEMENTS IN OTHER LIQUEFACTION PROCESSES

Element	Process: Stream: Coal: Concentration: Reference:	H-Coal Vacuum Bottoms Illinois Log <sub>10</sub> ppm Griffin et al. (1978)	Solvent Refined Coal Product Unspecified Percent of Feed Element in Stream <sup>a</sup> Jahnig and Magee (1975)	Synthoil	
				Product Western Kentucky Homestead Percent of Feed Element in Stream Schultz et al. (1977)	Effluent
Antimony		<1	2	-	-
Arsenic		1-2	4	-	-
Beryllium		1-2	58	-	-
Boron		3-4	80	-	-
Cadmium		<1	5	-	-
Chromium		2-3	2	37	65
Copper		1-2	78	48	75
Iron		>4	<1	31	74
Lead		1-2	3	-	-
Manganese		2-3	4	33	77
Mercury		<1	75	30	70
Nickel		1-2	6	-	-
Selenium		1-2	21	63	72
Tin		<1	14	-	-
Vanadium		2-3	7	-	-
Zinc		2-3	12	-	-

<sup>a</sup> Based on assumed yield of 75 percent (solvent refined coal), dry coal basis.

TABLE 9. SYNFUELS TRACE-ELEMENT CONCENTRATIONS IN LURGI RESIDUE AND LOSSES THROUGH LURGI AND HYGAS GASIFIERS

Element	Process: Stream: Coal: Concentration: Reference:		Lurgi Residue ("ash") Illinois 5 ppm		Lurgi Residue ("ash") Unspecified ppm		Lurgi Loss Through Gasifier Illinois 5 <sup>a</sup> Weight Percent <sup>b</sup>		Hygas Loss Through Gasifier Unspecified Weight Percent	
			Sather et al. (1975)		Griffin et al. (1978)		Sather et al. (1975) <sup>a</sup>		Attari et al. (1975)	
Antimony			0.3	0.2	1-10		91	81	33	
Arsenic			0.3	0.1	1-10		98	99	65	
Beryllium			21	13.7	10-100		6	—	18	
Boron			673	622	100-1000		79	—	—	
Cadmium			<0.3	<0.3	<1		—	—	62	
Chromium			570	755	100-1000		0	0	0	
Copper			273	239	10-100		0	0	—	
Iron			15 × 10 <sup>4</sup>	5.2 × 10 <sup>4</sup>	10 <sup>4</sup>		0	0	—	
Lead			210	71	10-100		32	—	63	
Manganese			320	200	>1000		0	0	—	
Mercury			0.01	0.02	<1		99.5	99.6	96	
Nickel			462	456	10-100		0	0	24	
Selenium			—	—	<1		—	—	74	
Tin			—	—	—		—	—	—	
Vanadium			181	301	100-1000		19	0	30	
Zinc			15.8	4.7	100-1000		29	0	—	

<sup>a</sup> Based on residue representing 9.43 percent and 11.1 percent of dry feed coal (from ash balance calculation).

<sup>b</sup> Weight percentage of trace elements in feed not found in ash-rich residue.

TABLE 10. SYNFUELS TRACE-ELEMENT CONCENTRATIONS IN SYNTHANE CONDENSATE AND PERCENTAGE OF FEED ELEMENTS IN RELATED GASIFICATION PROCESSES

Element	Process: Stream: Coal:	Synthane Condensate Illinois 6	Synthane Condensate Percent of Feed Element in Stream Baria (1975)	Synthane Condensate Percent of Feed Element in Stream Forney et al. (1975) <sup>a</sup>	Tar	Char	Fines
Concentration:	ppb						
Reference:	Sharkey et al. (1975)						
Antimony	—	—	—	0	0	203.1	2.0
Arsenic	30	—	0.06	0.2	3.0	116	4.3
Beryllium	—	—	—	0	0.2	138.4	4.2
Boron	—	—	—	75.7	0.9	95.1	0.6
Cadmium	—	—	—	0	1.4	404.1	10.4
Chromium	6	—	0.02	0	0.2	48	0.4
Copper	20	—	0.09	0	0.3	29.8	1.1
Iron	$3 \times 10^7$	—	—	—	—	—	—
Lead	—	—	—	0.1	1.2	656.4	5.1
Manganese	40	—	0.21	0.1	0.1	43.7	0.0
Mercury	—	—	—	39.9	19.6	15.7	1.2
Nickel	30	—	0.13	0.1	0.3	55.7	1.0
Selenium	360	—	9.23	11.1	0.5	310.5	9.9
Tin	20	—	0.70	0	0.2	68.9	2.6
Vanadium	3	—	0.005	0	0.1	53.4	0.9
Zinc	60	—	0.1	0.1	0.1	71.9	0.6

<sup>a</sup> Averages of three runs. Elemental balances range from 48 to 660 percent.

TABLE 11. REPRESENTATIVE LEVELS OF TRACE  
ELEMENTS IN SHALE OIL

<u>Element</u>	<u>Representative Level</u> (ppm)
Be	0.02
Hg	0.3
Cd	0.1
Sb	0.02
Se	0.4
Mo	2.
Co	1.
Ni	5.
Pb	20.
As	0.2
Cr	0.5
Cu	0.1
Zr	5.
B	2.
Zn	3.
V	1.
Mn	1.
F	1.
Fe	60.
Ti	15.
Al	15.
Ba	2.
Mg	7.
Ca	7.
Na	19.
Si	20.

TABLE 12. TRACE ELEMENT CONTENT IN SPENT SHALE

<u>Element</u>	<u>Representative Value</u> ppm, raw shale basis
Be	1.0
Hg	0.04
Cd	0.5
Sb	0.5
Se	0.3
Mo	10.
Co	16.
Ni	38.
Pb	34.
As	25.
Cr	100.
Cu	26.
Zr	43.
B	100.
Zn	35.
V	80.
Mn	250.
F	900.

TABLE 13. APPROXIMATE COMPOSITION OF TRACE ELEMENTS IN  
PROCESS WATER

	<u>In Situ</u> (mg/L)	<u>Above Ground</u> (mg/L)
Arsenic	0.3	0.09
Cobalt	0.4	0.01
Boron	0.3	0.06
Vanadium	1.2	0.01
Titanium	0.2	0.9
Iron	0.5	5 - 10
Molybdenum	0.5	0.1 - 0.3
Tin	0.1	0.05
Rubidium	0.2	0.01 - 0.4
Silicon	N.A.	4 - 7

N.A. = Not Available

TABLE 14. LEVELS OF MINOR ELEMENTS IN RETORT WATER

Element	Gas Combustion Simulated In-Situ Retorts					Indirect Heated Retorts	
							Paraho
Be	--	--	--				--
Hg	.01	.01	.39	.01	.02	--	--
Cd	--		--	.001	.003	--	--
Sb	.007	.007	.016			--	--
Se	.005	.01	.98			.096	0.7
Mo	.47	.1	--			.006	0.1
Co	.37	.07	.65			.005	.04
Ni	.26	1	--			.03	.2
Pb	.01	.1	--	.03	0.1	.002	.2
As	.26	6	6	2	0.3	1.0	1
Cr	.012	.02	.02	--	--	.007	.3
Co	.003	.007	--	.2	5	.16	.2
Zr	.02	.07	--			.003	--
B	.26	6	--			.44	5
Zn	.04	.4	.43	5	1	.045	.4
Li	--	.3	--			.006	1
V	1.2	.07	--			.002	.03
Mn	.023	.1	--			.019	.3
F	--	25	--			.3	7
Ba	.03	.05	.13			.09	2
Fe	.49	25	1			5.7	5



TABLE 15. TRACE ELEMENTS IN WATER EXTRACTED FROM  
EXPERIMENTAL IN SITU RETORT TEST AREA (ppm)<sup>a</sup>

Element	Site 9 production wells <sup>b</sup>	Site 9 observation wells <sup>c</sup>
Uranium	1.082	0.064
Lead	0.0356	0.1924
Mercury	0.00152	0.00086
Cadmium	0.0035	0.00175
Molybdenum	4.1	1.0411
Strontium	0.56	0.3544
Bromine	0.48	5.7598
Selenium	0.007	0.00475
Arsenic	0.1487	0.0189
Zinc	0.774	0.0904
Copper	0.087	0.0417
Nickel	0.329	0.11125
Cobalt	0.0146	0.0155
Manganese	0.0503	0.1562
Chromium	0.0149	0.0075
Vanadium	0.0779	0.05175
Aluminum	4.779	2.1228
Fluorine	33.25	31.99
Boron	30.17	41.03

<sup>a</sup>From Jackson et al. (1975).

<sup>b</sup>Average of analyses from 9 wells.

<sup>c</sup>Average of analyses from 10 wells.

## IMPACTS FROM POLYCYCLIC ORGANIC MATERIALS

Polycyclic organic materials (POM) are organic compounds that contain several benzene-ring structures. The general term POM is used to include all multi-ring organic compounds. They are formed during the conversion of coal, retorting of oil shale, upgrading of liquid products, and combustion of synthetic fuels. The most important of them are listed in Table 15 (Kornreich, 1976).

The conversion of coal, retorting of oil shale, upgrading of fuel, and combustion of synfuels involve elevated temperatures to levels at which POM can form. Both epidemiologic and animal experimentation data indicate that many of the POM compounds are carcinogenic, mutagenic, and teratogenic. The concern about their health effects is based primarily on two types of studies:

- (a) Epidemiologic studies which have indicated an increased risk of cancer to workers routinely exposed to the products of coal, such as workers in coal gas, coking, coal hydro-generation industries, street pavers, and asphalters.
- (b) Laboratory studies using bacteria, human cell culture system and whole animals to determine the carcinogenicity of various coal products.

Redmond, in a series of studies (Energy Research and Development Administration, 1976), has attempted to correlate the risk from lung cancer within a coke plant with work areas, duration of employment, and race. The study has included about 60,000 steel workers employed at seven plants. It has been found that men who worked for 5 years or more in the areas where exposure to coke oven emissions was the greatest (top of ovens), experienced about 900 percent excess lung cancer mortality rates as compared to other steel workers. As expected, the excess cancer mortality rates were found to be lower for coke workers at cleaner localities and/or shorter periods of employment. The results in respect to racial differences have been largely inconclusive. Whites have been observed, however, to have lower cancer excess mortality rates for those employed in the total coke oven area as well as for the most polluted areas.

Mazumdar (Mazumdar et al., 1975) has been able to develop a relationship between exposures to coal tar pitch volatiles (CTPV) and excess cancer risks to exposed coal workers. He has applied the risk factors found by Redmond to exposure data developed by the Pennsylvania Department of Health at ten coke oven installations (Fannick et al., 1972). The data have revealed considerable differences in exposures to CTPV for the various coke oven jobs. Exposures varied from  $3.23 \text{ mg/m}^3$  for the high average exposure, to  $0.50 \text{ mg/m}^3$  for the low average. The author has concluded that both the level and length of exposure are related to cancer development, particularly lung cancer. The latent period between first exposure to CTPV until death has varied between 10 and 14 years, averaging 25 years. No excess cancer incidence has been observed for workers exposed for an average of 30 years to the established threshold limits of  $0.2 \text{ mg/m}^3$  of CTPV.

TABLE 16. CHEMICAL CARCINOGENS IN COAL OR COAL-DERIVED MATERIALS

CHEMICAL	RELATION TO COAL	CARCINOGENIC ACTIVITY
<u>POLYNUCLEAR AROMATIC HYDROCARBONS</u>		
Benzo (a) pyrene	Identified in coal tar, coal tar pitch fumes, coal combination stack effluents, workplace atmosphere at coking plants and gasworks retort houses.	Highly carcinogenic in animals (9 species) by oral, dermal or intratracheal administration.
Dibenz (a,h,) anthracene	Identified in coal tar and soot.	Highly carcinogenic in animals (6 species) by oral, dermal, intratracheal or subcutaneous administration. Effective in lower dose than B(a)P.
Benz (a) anthracene	Identified in coal tar, coal tar pitch, soot, creosote, flue gas from coal furnaces and other coal fired installation, workplace atmosphere or gasworks retort and a coal-fired power plant.	Highly carcinogenic in mice by oral, dermal and subcutaneous administration.
Dibenzo (a,l) pyrene	Identified in coal tar.	Highly carcinogenic in mice and hamsters by dermal and subcutaneous injection.
Indeno (1,2,3,-cd) pyrene	Identified in coal tar, coal tar pitch, and soot.	Highly carcinogenic in mice by dermal and subcutaneous administration.
Benzo (b) fluoranthene	Identified in coal tar and soot.	Carcinogenic in mice by dermal and subcutaneous administration.
Benzo (j) fluoranthene	Identified in coal tar, soot and creosote oil.	Carcinogenic in mice by dermal application.
Dibenzo (a,h) pyrene	Identified in coal tar pitch.	Carcinogenic in mice and rats by dermal and intratracheal administration.
Benzo (a) pyrene	Identified in coal tar, coal tar pitch, soot, creosote, domestic coal combustion stack effluents and gasworks retort houses.	Weakly carcinogenic in mice by dermal application.
Chrysene	Identified in coal tar, coal tar pitch, soot and bituminous coal.	Weakly carcinogenic in mice by dermal and subcutaneous administration.

TABLE 16. CHEMICAL CARCINOGENS IN COAL OR COAL-DERIVED MATERIALS (CONTINUED)

CHEMICAL	RELATION TO COAL	CARCINOGENIC ACTIVITY
<u>POLYNUCLEAR AZA-HETEROCYCLIC COMPOUNDS</u>		
Benz (c) acridine	Identified in coal tar pitch and domestic coal combustion stack effluent.	Carcinogenic in mice by dermal application and bladder implantation.
Dibenz (a,h) acridine	Identified in domestic coal combustion stack effluent and air polluted by coal tar pitch.	Carcinogenic in mice by dermal and subcutaneous administration.
Dibenz (a,j) acridine	Identified in domestic coal combustion stack effluent.	Carcinogenic in mice by dermal and subcutaneous administration.
<u>AROMATIC AMINES</u>		
2-Naphthylamine	Identified in coal tar, coking pitch and atmosphere of gas retort houses.	Demonstrated human bladder carcinogen. Carcinogenic in mice, hamsters, dogs and monkeys by various routes of exposure.
1-Naphthylamine	Identified in coal tar.	Weakly carcinogenic in mice by oral administration.
<u>BENZENE</u>	Identified in coal tar distillates; produced commercially from coal tar.	Associated with increased incidence of leukemia in humans.
<u>TRACE ELEMENTS</u>		
Arsenic	Identified in coal, Synthane gasifier condensate, chars, and fines.	Causal relationship between human skin cancer and heavy exposure to inorganic arsenic in drugs, drinking water, or occupational environment. Human respiratory carcinogen.
Beryllium	Identified in coal, Synthane gasifier chars and fines, and from oil produced by catalytic hydrogenation of coal.	Elevated risk of lung cancer in beryllium workers. Beryllium sulfate, beryllium oxide, beryl ore, bertrandite and beryllium metal particles. Carcinogenic in rabbits (intravenous), rats (inhalation), or monkeys (inhalation).

Another study (Oak Ridge National Laboratory, 1977) has measured CTPV and benzo(a)pyrene profiles in a coke manufacturing plant. The results have been similar to those reported by Fannick (1972). CTPV values for the more contaminated areas have varied between 0.0313 to 1.67 mg/m<sup>3</sup>, and for the cleaner areas between 0.0072 to 0.322 mg/m<sup>3</sup>. The levels of benzo(a)pyrene (BaP) concentrations atop the oven have been about 18 µg/m<sup>3</sup> or about 100 to 1,000 times greater than the typical levels found in urban atmospheres. Workers at the less contaminated areas (i.e. side oven) have been exposed to BaP concentrations of about 7 µg/m<sup>3</sup>. However, synfuel plants differ considerably from the other workplaces studied. Processing is accomplished in closed systems so that routine contacts with labile chemicals will be very small.

The only reported incidence of cancers among occupational workers of coal conversion plants has been published by Sexton (1960). His information pertains to a coal liquefaction pilot plant at Institute, West Virginia, that was in operation from 1952 until the mid-1950's. Skin contamination rather than inhalation exposure was the cause of the excess skin cancer rates that were observed by Sexton (1960). Between 1954 and 1959, 42 cases of skin lesions, which were diagnosed as precursors of skin cancers, were treated among the 259 employees. In addition, there were 11 probable and five verified skin cancers. Analysis of the liquid materials produced in the plant identified a number of carcinogenic materials. These rates are 16 to 37 times greater than found in the population of the United States and of West Virginia. Exposure periods of the men to the chemicals varied from several months to several years, since some of the workers had been exposed to similar chemicals in previous laboratory work. The effectiveness of the industrial hygiene program conducted at the facility on workers during the operation of the plant indicated skin contamination in spite of the cleaning procedures and protective measures. Only limited conclusions can be drawn from these data, namely, that skin contamination by coal liquefaction products induces skin cancer. No correlation between exposure and incidence of skin cancer has been determined. Also, the data are not inclusive concerning the incidence of other kinds of cancer.

An attempt was made to correlate the lung cancer incidence observed from several epidemiologic studies with coking temperatures (U.S. Department of Energy, 1979). It was observed (Energy Research and Development Administration, 1976; National Institute for Occupational Safety and Health, 1972) that the formation of carcinogenic material and the resulting cancer incidence increased rapidly between 450°C to 1,500°C. Only small numbers of excess lung cancer incidence were found below 450°C (about 27%). The excess incidence increased to 83% at 1,000°C, and to 800% at 1,500°C.

Experimentation on animals has showed similar findings to those observed from epidemiologic studies, namely, that various organs can develop cancer from coal products, that several coal extracts are carcinogenic, and that coal tars produced at higher temperatures are considerably more carcinogenic than those produced at lower temperatures (Energy Research and Development Administration, 1976).

Studies on the potential carcinogenicity of crude and refined shale oils are much more limited than for coal conversion products. Carcinogenic products have been identified in some crude and refined shale oils (Yen, 1979; Berenblum and Schoental, 1943; Berenblum and Schoental, 1944). The few animal and epidemiologic studies reported on oil shales and some of their components and commercial products indicate the possibility of carcinogenic risks (Twort and Twort, 1931; Veldre and Yanes, 1977).

Studies of workers in petroleum refineries and in the petroleum industry have not yielded evidence of increased cancer risk (Veldre and Yanes, 1977).

The available information on potential mutagenicity of trace contaminants derived from coal conversion and oil shale retorting and their products is also very limited. The data are confined to identification of some potential mutagens by tests on cell systems including human cells. Most of the compounds are also carcinogenic, and it has been concluded that the correlation between carcinogenicity and mutagenicity is very high.

In order to assess the carcinogenic risks of synfuel processes and products, we need additional information. This would include:

- (1) Knowledge on how to measure all POM's in emissions (Allen et al., 1978). Usually, only one POM, namely benz(a)pyrene, is measured as an indicator to all POM's. For true comparison of different sources, and for complete assessment of individual sources, it is important to measure all POM's.
- (2) Better understanding about the formation mechanisms, distribution in process streams, emission characterization, and effectiveness of emission controls to reduce the various POM emissions (Allen et al., 1978; Pack, 1979).
- (3) Quantitative data are needed about the dispersion, persistence and reaction products resulting from transformation processes of POM's in the environment.
- (4) Additional information is needed not only to assess the carcinogenic health risks of synfuel processes but also about the mechanisms of cancer formation by POM. There is a lack of knowledge even about differences in the metabolism of the carcinogenic POM in target tissues (lung, skin, intestine) and non-target tissues (liver and others).

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## OPEN FORUM

DR. SHIOTSUKA (Brookhaven National Laboratory): My question is directed to Dr. Dalbey. I would like to know if you were able to estimate the amount of aerosol loss at the cone that you installed within the chamber and at the two perforated aluminum pans and also whether you use any catch pans in your chamber under the animal cages?

DR. DALBEY (Oak Ridge National Laboratory): Had I been given a choice, I would not have put the baffles in. If I had chambers with a tangential entry of feed air at the top, for example, I would much prefer to have the octopus L-nuts at the bottom such as Carpenter used at Lovelace. We do not have that kind of chamber, however, so we felt constrained to go to the baffle system. I really don't know how much aerosol is trapped up there. If we operate at a concentration of less than 16 milligrams per liter or down in the range where we operate more at about 2 milligrams per liter, we see virtually no deposition on the walls of the chamber, on the cages, on the animals, or anywhere. We've looked at the baffles and at the cone on top and have seen just a few drops here and there. There apparently is a very minor amount of deposition. No, we do not use catch pans. With the advent of some of the new style chambers in use at Hazelton Laboratories or at Battelle, catch pans in inhalation chambers may be coming into use. We don't use them as yet. We may consider putting them in our chamber if we can be satisfied that we still get good distribution of the aerosol.

DR. COURI (Ohio State University): I'd also like to ask a question on an operational level. I was interested in the composition of the VEESS material in the field compared to what is produced in the laboratory. In the field, is the smoke cloud generated by dropping raw fuel on the exhaust manifold or is it a product of the total fuel combustion of the vehicle?

MR. WEIMER (U. S. Army Chemical Systems Laboratory): Mr. Callahan has a poster session on the pump used in the generator system and he can describe all of this for you.

DR. COURI: Where is Mr. Callahan?

MR. CALLAHAN (U. S. Army Chemical Systems Laboratory): In the VEESS systems vehicle, for example, the M60A1 tank has two twelve cylinder engines on either side and has exhaust manifolds coming from either side that are used independently for smoke generation. The vehicle driver can selectively trigger a stream of diesel fuel to be sprayed on either hot exhaust manifold at a rate up to one gallon per minute. The manifold temperature has to be about 1150° Fahrenheit at the beginning of the smoke making operation or you don't get maximal volatilization. You do get a cooling effect on the manifold and it's lowered about 50% down to about 500° Fahrenheit. The principle of the operation is that with the drop in exhaust manifold temperature, additional smoke comes from the engine exhaust and the total smoke is a mixture of the two sources.

DR. COURI: Thank you. That accounts for the similarity of patterns that you got between the field and the laboratory samples. I was under the impression that was a regular combustion process that made the smoke.

MR. WEIMER (U. S. Army Chemical Systems Laboratory): We have been a little concerned about the presence of the particulates from the diesel engine itself which probably would constitute a relatively minor portion of the total mass that the animals are exposed to. We also are concerned about the presence of oxygen in the generator. We're using pure nitrogen right now. We recently found that if we add 10% oxygen we end up with a higher amount of aldehyde content in the aerosol as it comes into the chamber. This is a problem that we're wrestling with at the present time.

DR. COURI: One more minor point. The aromatic compounds or volatiles that are measured are on the particle? They're trapped on the aerosol particles. Is that correct?

MR. WEIMER: What I described was the particulate phase. Most of the analytical effort that's been made has been on the particulate phase so far, since that will be the principal contaminant that the animals will inhale. We estimate that around 15% of what the animals will inhale is in the gaseous phase. There are some aldehydes and a few other materials that are being characterized, but the total amount is relatively small.

MR. WANDS (The MITRE Corporation): I'd like to ask two questions of Dr. Dalbey also. First of all, don't you have a problem with the potential for fire in your laboratory? I did not notice in your rough diagram any flashback protection from that hot source.

DR. DALBEY (Oak Ridge National Laboratory): When we first started planning this study, we were concerned with the fire hazard too. We didn't want to have an explosion in our lab. We were assured by people who used flame throwers in diesel fuel smoke screens that they had not been able to ignite the aerosol. We've done our own experimentation to check the hazard and found that when you get concentrations of fuel of around 60 milligrams per liter, the aerosol could be ignited with an electric spark. We don't have a real safety problem here.

MR. WANDS: Second question, please. This aerosol is going to condense on the animal's fur. Are you concerned about the problem of preening in consideration of the total dose delivered to the animals?

DR. DALBEY: Yes, but I can't give you any hard numbers on that point. We are concerned about that factor since we are giving the animals a whole body exposure. Subjectively, we see no evidence of any deposition on the skin at concentrations that are around normal working levels. That's not to say there isn't any deposition because I'm sure there must be. That's one of the reasons why we do want to do a deposition study and look at the amount which is deposited on the skin after an exposure which might give us some index of how much material the animals might get by that route.

MR. CALLAHAN (U. S. Army Chemical Systems Laboratory): I have just finished some acute studies with this particular fuel at concentrations of 35 milligrams per liter. You really had no problem with preening. At this concentration you did get an oily deposit on the fur but the toxic effects seen during the 14-day observation period did not appear to be related to the dose added by preening in B6C3F1 mice, Fischer rats, Sprague-Dawley rats and guinea pigs.

DR. DALBEY (Oak Ridge National Laboratory): I might add to this point. We were really surprised to see very little deposition of fuel droplets on the walls of the chamber when the smoke was so dense that one couldn't see through the chamber during a six-hour exposure. It was quite a pleasant surprise for us.

DR. MacFARLAND (Gulf Science and Technology Company): Dr. Dalbey, what was the volume of your chamber and the total flow rate through it?

DR. DALBEY: The volume of the chamber depends on whether you want to include the cone sections in the estimate. It's roughly 2 meters high in the working area where the animals are and about a meter square in cross section. We operate with a 15 cfm total flow through the chamber.

DR. MACH (Aerospace Corporation): Is there any difference between the toxicologic effects of low levels of smoke and low levels of the fuel coming in as an aerosol? Is smoke particularly dangerous or would the fuel be as toxic?

DR. DALBEY: We're not dealing with the smoke. Smoke to me is a combustion product. In our study we are dealing with an aerosolized fuel and that's what we are exposing the animals to.

DR. MACH: If you took the diesel fuel and just sprayed it through a fine nozzle could you produce the same white looking aerosol as you have in the system? Could you get concentrations of 15 milligrams per liter for example?

DR. DALBEY: We could probably produce a concentration of 15 milligrams per liter, but I think the particle size would be much larger than we are producing in our generation system. We're working with very fine particles about one micron in size and in the lower concentration exposures, the aerosols are much smaller than that. I would be extremely surprised if you could get such a small particle size with diesel fuel using a nebulizer.

DR. BACK (Air Force Aerospace Medical Research Laboratory): I'd like to change the subject a little bit and ask a question of Dr. Thomson. Your dog study data are quite elegant. Did you look at the action of your compound on smooth muscle?

DR. THOMSON (U. S. Army Chemical System Laboratory): No, we didn't.

DR. BACK: That compound is a smooth muscle relaxant, acting directly on smooth muscle and probably the blood pressure drop you observed was caused by smooth muscle relaxation.

DR. SHIOTSUKA (Brookhaven National Laboratory): Dr. Thomson, were you able to conduct cardiac output studies on your dogs and in the studies that you reported today, were the dogs conscious or anesthetized?

DR. THOMSON: They were anesthetized with sodium pentobarbital. We didn't have the equipment to measure the cardiac output but we measured heart rate and didn't see a change. We also didn't see any change in the EKG of the treated dog. I had wondered whether there would be an increase in cardiac output but I was unable to measure that.

MR. VERNOT (University of California, Irvine): Mr. Weimer, you indicated that in the acute test the phosphorus smoke was much more toxic to guinea pigs than to rats and I wonder whether you determined whether this was due to bronchospasm in the guinea pig or was it due to the irritating effects of the material itself?

MR. WEIMER (U. S. Army Chemical Systems Laboratory): Our pathologists looked at animals and they didn't see any evidence of bronchospasm. That was the first thought we had since it did appear to be an allergenic response to the exposure to acid mist, but we were unable to find a specific cause.

MR. VERNOT: I have another question for Dr. Dalbey. How did you sample your aerosol for further analysis by gas and liquid chromatography?

DR. DALBEY (Oak Ridge National Laboratory): This aerosol was collected on Cambridge filter pads which were then extracted with hexane.

MR. VERNOT: Was there any opportunity for loss of the material from the filter between sampling and analysis?

DR. DALBEY: That's one point I didn't bring up when I showed the picture of the slide that had the VEESS aerosol on it that was generated in the field. When that aerosol sample was obtained, the filters were located in a fixed position and after some effort, the tank was placed just upwind from those filters. It was a hot day, the air was rising up and it was not a good sampling trip. There were periods of time when the filters were not in the smoke screen and the smoke was 50 feet up in the air or someplace else, but the pump was still running. That was another reason for saying there may have been time when some of the low boiling compounds could have eluted off the particulate on the filter pad and we never would have seen them. In our chambers we don't run into that problem of not being able to sample properly. That is an important consideration since we are dealing with a dynamic aerosol.

MR. VERNOT: Dr. Salmon, your review of the possible toxic effects of the various synthetic fuels was excellent and exhaustive. I wonder whether any group at NAS is considering the broader environmental impact of coal derived synthetic fuels such as the increase in acid rain effects if the use of these fuels is increased significantly.

DR. SALMON (National Academy of Sciences): There is a committee looking at all the ecological effects.

DR. DUNGWORTH (University of California, Davis): Dr. Dalbey, how are you going to use the concentration times time data from the acute mortality studies? With ozone we find that it really isn't relevant at sublethal concentrations.

DR. DALBEY (Oak Ridge National Laboratory): I would like to have my statistician standing by my side right now like a lawyer to correct any mistakes I might make. The CT values were used with the methodology of designing our repeated exposures. We are not saying that the CT product is going to hold true for other endpoints. We wanted to see if that would be a viable consideration in our experimental design for Phase II of our study which is the repeated exposure phase. It gets statistically complex in terms of how we arrange our array of exposure concentrations, duration and frequency.

DR. DUNGWORTH: I think that is important to realize that with sublethal exposures, it's concentration which is the principal determinant of effect and you should build that into your exposure regimens.

DR. MASTROMATTEO (Inco Limited): Dr. Salmon, there is fairly good evidence that shale oils are skin carcinogens and heavy petroleum oils could also be carcinogens in chronic skin exposure. Was this taken into account in the committee's evaluation?

DR. SALMON (National Academy of Sciences): Yes, the committee considered the data of skin carcinogenicity found in mice during animal experimentation but we don't have sufficient evidence yet to try to really assess the impact of shale oils on humans.

DR. HODGE (University of California, San Francisco): I'd like to address a question to Dr. Newell. In summing up your discussion, you referred to some factors like duration, frequency, and the point at which there would be no severe or permanent disability that enter into decisions on short-term exposure limits and then you said something about the least risk to health. I must not have been listening closely enough because I missed the point of the last factor about risk to health. Would you elaborate on this point, please?

DR. NEWELL (National Academy of Sciences): The committee attempts to look at all the available data and frequently that is chronic and long-term exposure data. Then, because they are trying to recommend short-term limits if there are short-term exposure data that have dose-related effects, they try to extrapolate this information and relate it to the short-term human exposure. Admittedly, there's a big difference between a single exposure, whether it's for a few minutes or a couple of hours as opposed to a chronic exposure, but sometimes chronic exposure data is all that's available and that is when extrapolation is difficult.

DR. HODGE: How was the least risk choice made?

DR. NEWELL: That was done on the basis of using the exposure level where you see either no effect or the least effect and then, depending upon the reliability of the data, the committee may throw in some safety factor of ten or a thousand fold. You know that we occasionally do that.

DR. HODGE: Thank you.

DR. BACK (Air Force Aerospace Medical Research Laboratory): A statement about nomenclature, Dr. Salmon, about synthetic fuel. Synthetic fuel can mean two different things. When people ask me about synthetic fuels I talk about compounds that are built from scratch and not the difference between shale derived and petroleum derived. They are actually compounds that are synthetically formed in a retort or in a similar reaction vessel. We are already having problems when we get questions about synthetic fuels. When I speak of synthetic fuels I am referring to compounds like JP-10, RJ-5 which are norbornane dimer derivative which are pure compounds or simple mixtures. They are not gamisches of things like that which comes out of shale. They are pure chemical entities and I think we ought to uniformly call synthetic fuels only those that really are and call fuel extract from natural source something like alternate fuels or some other suitable precise term.

DR. SALMON (National Academy of Sciences): I agree with you, Dr. Back. I didn't want to confuse the audience, but right now the definition of synthetic fuels according to the DOE energy plan includes also heavy oils, oil sands and many others that are natural substances, so the definition has been increased by governmental fact.

DR. NEWELL (National Academy of Sciences): I just want to respond to Dr. Back's comment. Dr. Back, in about 5 or 10 years from now, what total volume of fuel production do you think these true synthetic fuels will be in relation to those derived from natural sources? The objectives are to be able to put out anywhere from two to ten million barrels of crude fuel a day.

DR. BACK (Air Force Aerospace Medical Research Laboratory): Are you asking about the ramjet fuels?

DR. NEWELL: Yes, those that are the true synthetic fuels by your definition.

DR. BACK: The volume is much less but the amount of work you do with them is exactly the same.

DR. NEWELL: I was trying to come up with a suggestion. Maybe we ought to call those specialty synthetics as opposed to what the press or DOE is going to call synthetics derived from natural sources.

DR. BACK: Alcohol may be a synthetic material. Methanol can be used as an alternate fuel but I'd rather have them called alternate fuels if they are going to be used in bulk quantities as fuels. The Navy is deriving diesel fuel marine from shale and the Air Force is deriving JP-4 from shale oil and commercially, I suppose JP-8 or commercial jet fuel will ultimately come from shale.



DR. NEWELL: You have raised a good point.

DR. BACK: I'd prefer to call them alternate fuels.

DR. NEWELL: We ought to try to be more careful in our definitions and identifications.

COL. CARTER (Air Force Aerospace Medical Research Laboratory): This seems to be a somewhat ridiculous problem but it is very confusing. We really ought to come to some agreement on terminology for either petroleum or non-petroleum derived fuels. It is much more descriptive to refer to a non-petroleum or shale derived JP-4 or gasoline.

DR. MacFARLAND (Gulf Science and Technology Company): In Dr. Salmon's presentation he showed statistics that indicate that traumatic accidents and coal workers pneumoconiosis (CWP) are things to worry about in synthetic fuel production; yet a great deal of his discussion was given to the question of the vast array of trace amounts of highly toxic heavy metals that are found in shale materials and in flyash from coal. It always seems to me that the concern about this is undue at least in certain places where these things may turn up as contaminants. As an experimental toxicologist, the best kind of study that I know how to do is a long-term repeated exposure study in which animals receive a daily inhalation burden of the material. I have done some long-term two year studies in primates and rodents and I've studied flyash and raw shale dust and spent shale after retorting and despite the most extensive histopathology on 40 plus tissues from each of the animals we are unable to demonstrate any kind of systemic intoxication that could be attributed to heavy metals.

MR. WANDS (The MITRE Corporation): Dr. Salmon, about thirty years ago, Union Carbide had a pilot plant for coal conversion. How do these modern conversion processes compare with that in terms of the temperatures required, materials produced, and how applicable are their observed health effects to our modern situation? Do we already have a lot of the answers that we need?

DR. SALMON (National Academy of Sciences): I have to admit that I'm not very familiar with the Union Carbide process; I've seen it but I've not seen an analysis of the products. We have many new processes and none of them has really been evaluated in such a way that you can come up with conclusive answers at this time. The synthetic fuel processes are so different from each other that you may come up with some generalizations, but eventually you need to do evaluations for each specific one at their sites. The processes that are approaching finalization where a risk assessment has been done for purposes of getting permits are the ones I believe when I see the results. There is too much being published which is purely speculation that adds to the confusion.

DR. MacFARLAND (Gulf Science and Technology Company): In that connection, I'd remind you of references made to the South African coal conversion activity. I'd remind you of the fact that the Nazis fueled their planes in World War II with fuels derived from coals, that there is a very viable and long existing shale oil conversion activity going on in Estonia, and that the Scottish shale industry grew up in the 19th century and flourished and finally died out essentially because

they were running out of shale. I share the same concerns that are expressed about reinventing the wheel.

DR. NEWELL (National Academy of Sciences): Dr. MacFarland, your concerns are certainly well founded. I'm sure you realize the Department of Energy has the responsibility to attempt to assure an understanding of the health risks involved in these various fuel extraction and refining processes. There is a group concerned just with a health effects research program (HERP) within DOE that is looking at all the data being accumulated and thinking about the kinds of data that should be accumulated to try to make judgements about health standards.

## SESSION IV

### EPIDEMIOLOGY OF INDUSTRIAL SOLVENTS

#### Chairman

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SUMMARY OF EPIDEMIOLOGICAL STUDIES OF  
TEXTILE COMPANY EMPLOYEES EXPOSED TO  
METHYLENE CHLORIDE

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G. G. Bond, M.P.H.

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INTRODUCTION

Recent studies by a team of Dow investigators illustrate some of the epidemiologic techniques used to study work-place exposures to industrial chemicals. The studies were conducted during 1977-1978 in textile plants owned by the Celanese Fibers Company. Detailed results will be published in a forthcoming issue of the Scandinavian Journal of Work and Environmental Health, so this paper should be considered an overview and not a substitute for the complete report. Principal investigators were Lyman Skory, M.S., Study Director and Senior Environmental Specialist, Carl Strande, M.D., Clinical Toxicologist and M. G. Ott, M.S., Statistician.

Methylene chloride is a widely-used industrial solvent with the unusual property of being metabolized to carbon monoxide, thus leading to increased carboxyhemoglobin levels in man as well as in laboratory animals (Stewart et al., 1972). Epidemiologic data on results of chronic workplace exposures have been negative. A well organized, recent, large-scale epidemiologic study of Eastman Kodak workers has shown no deleterious health effects in workers with long term exposures to methylene chloride time-weighted-averages (TWA) in the ranges of 30 to 125 ppm (Friedlander et al., 1978). Nevertheless, a National Institute of Occupational Safety and Health (NIOSH) criteria document (NIOSH, 1976) has recommended lowering the existing TWA of 500 parts per million (ppm). Epidemiologic data to support this action are lacking, although the American Conference of Governmental Industrial Hygienists (ACGIH, 1980) recommends a standard of 200 ppm TWA.

In order to expand the previous studies and to further study the oxygen dissociation kinetics of methylene chloride in humans, the Dow researchers conducted a series of health studies on textile company employees who had experienced methylene chloride exposures higher than had occurred in the Eastman Kodak plants. The studies were accomplished using a variety of epidemiologic methods, including a mortality analysis by the retrospective cohort technique, a questionnaire survey of morbidity, and a cross-sectional study of clinical laboratory results. Additional clinical studies were also carried out. These included blood oxygen transport measurements and continuous electrocardiogram (EKG) monitoring of selected men.

## MATERIALS AND METHODS

A textile plant in South Carolina which since 1954 has used methylene chloride to produce cellulose triacetate fibers was chosen as the source of a study population. A plant in Virginia which produced acetate fibers and had similar chemical exposures except for methylene chloride provided the comparison population. All study subjects were employee volunteers. A questionnaire regarding recent and long-term health status was distributed to both groups, and clinical laboratory tests were performed on blood specimens from volunteers from both plants. Continuous 24-hour EKG monitoring was by Mini Holter recorders attached to 50 white males from the two plants selected because they were considered at risk to coronary heart disease based on their medical histories. Studies of carbon monoxide in blood, and of the effects of methylene chloride on oxygen transport, were performed on smoker and nonsmoker volunteers from both plants. The epidemiologic studies used established methodology, including comparisons of answers from questionnaires in Mantel-Haenszel (1979) 2x2 contingency tables, or comparisons of mean values of laboratory tests results by the Student's-t test (Colton, 1974). Death certificate data were also collected; using person-years accumulated by the exposed and control cohorts, comparisons were made with expected deaths based on U. S. white male mortality rates.

Comprehensive industrial hygiene surveys were carried out at both the study and comparison plants to learn existing exposure ranges not only for methylene chloride, but for other chemicals as well. For this purpose, more than 500 personnel monitoring and area samples were analyzed. Contaminants such as oil mist and carbon monoxide were present in the working environments, but at both plants were detected at levels so low as to be judged clinically silent. Exposure to acetone vapor, however, occurred at significant levels in both plants; so the comparison group, which was not exposed to methylene chloride, did, in fact, have exposure to acetone. Generally, acetone levels were highest where methylene chloride was lowest. In addition, in triacetate production, methylene chloride exposure was accompanied by exposure to methanol at a ratio of about 1 ppm of methanol to each 10 ppm of methylene chloride. TWAs of methylene chloride encountered in the study plant varied depending on job assignment; TWA concentrations of acetone ranged from 10 to 800 ppm (to 1470 in the comparison plant).

## RESULTS AND DISCUSSION

The comparison plant had been purposely chosen to provide controls from a textile manufacturing facility which did not use methylene chloride. This was accomplished, but there were additional dissimilarities between the two factories so statistical comparisons between them were not always appropriate. In the study population cohort, the observed number of deaths in white males was about what would have been expected using all U. S. mortality for indirect adjustment of the expected deaths, yielding a standard mortality ratio (SMR) of 102% (37 deaths vs. 36.2 expected). This SMR is somewhat higher than is sometimes encountered in working populations. By contrast, the SMR of 44% (25 vs. 55.2 expected) for white males in the comparison group was abnormally low, even for working populations. The authors did not consider the observed differences in mortality experience between the study and comparison groups to be due to

methylene chloride exposure, partly since cause-specific analyses of deaths in white males (the predominant group) revealed fewer than expected deaths from malignancies or from cardiovascular disease in both groups.

Few differences were encountered in reported illnesses according to the self-administered questionnaire. Recent colds and fever reported by the study population were considered due to an outbreak of upper respiratory disease experienced in that geographic area.

Analysis of results of laboratory studies revealed the expected differences in blood indices between whites and nonwhites and males and females, and, as expected, increased levels of carboxyhemoglobin were associated both with cigarette smoking and with methylene chloride exposures. An increase in mean corpuscular volume (MCV) as has been reported previously (Stewart et al., 1972) in cigarette smokers was also observed, but the MCV of those with methylene chloride exposure was within normal limits. Except for total serum bilirubin, clinical chemistry results were either within normal limits or had no pattern consistent with progressively higher concentrations of methylene chloride. Overall, the mean total bilirubin levels in white males of the study group was 0.7 mg % while it was 0.5 mg % in the comparison group, and there were more high than low exposure methylene chloride workers with total bilirubin in the abnormally elevated ( $>1.1$  mg %) range. Reasons for this apparently dose-related elevation of serum bilirubin were not apparent, since there was no evidence of red cell destruction nor were there other abnormal tests of liver function.

Continuous EKG monitoring has not been reported previously as a method for industrial health surveillance. Using the Holter Monitors, satisfactory 24-hour tapes were obtained from all but 3 of 50 white male volunteers on the first try, and acceptable tapes were obtained from 2 of those 3 on a second 24-hour run. Since volunteers were purposely recruited from high risk groups, it is not surprising that many (67% and 62% in exposed and control groups, respectively) experienced ventricular ectopic activity (VEA) of various degrees. VEA, which might indicate cardiac sensitization, did not correlate with methylene chloride exposure. Other EKG findings included ST depression in two of the comparison and one of the study group volunteers.

A total of 266 volunteers participated in the studies on oxygen transport, including smokers and nonsmokers from both the study and comparison plants. Measurements were made of blood carboxyhemoglobin, alveolar air carbon monoxide concentrations and the " $P_{50}$ ", which is the partial oxygen pressure required to keep 50% of blood oxygen carrying capacity saturated with oxygen. Blood samples for oxygen and carbon monoxide analyses were drawn before and after work shifts.

As expected, smokers and methylene chloride exposed workers had higher blood carbon monoxide levels and lower  $P_{50}$  levels than did nonsmokers and controls. The historical normal mean  $P_{50}$  is 26.6 mm Hg with a standard deviation of 1 mm Hg (Neville, 1977). In this study, higher mean values (greater than 28 mm Hg) were found in nonsmokers in the comparison plant. The  $P_{50}$  of other groups, including those exposed to methylene chloride was 26 mm Hg or lower. Thus, results from the exposed population and from smokers were within the historical range but were less than those from the comparison population. When

corrected for the effect of blood CO, significant differences from normal were again encountered only for comparison plant workers, particularly nonsmokers. Not surprisingly, overall oxygen affinity was higher (and hence  $P_{50}$  levels lower) in study than in comparison samples, in men than in women, in smokers than in nonsmokers and after work shifts rather than before.

Using regression analysis, an indirect effect (via metabolism to carbon monoxide) of methylene chloride on  $P_{50}$  was established, but this was less than the observed differences between smokers and nonsmokers. Interestingly, exposure to acetone in the comparison plant was associated in a crude dose-response fashion with significant elevations of  $P_{50}$ . However, all of the observed changes in the blood oxygen transport system were considered well within the system's regulating capacity.

### SUMMARY AND CONCLUSIONS

These studies involved collection and analyses of both morbidity and mortality data. The overwhelmingly negative results confirm those of a previous epidemiologic investigation by researchers at Eastman Kodak and reinforce confidence in existing permissible exposure levels. Overall, methylene chloride exposure was associated with increased blood carboxyhemoglobin, with decreased oxygen saturation, and with slight elevations of serum bilirubin. The first two effects were expected; there was no ready explanation for the latter.

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EPIDEMIOLOGIC STUDY OF DRY CLEANERS  
EXPOSED TO PERCHLOROETHYLENE

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Because I have been told that a general discussion of epidemiology might be at least as useful to the people attending as the specific points raised in this particular study, I will start with a few general remarks.

I assume most of you have a strong background and interest in experimental science; so it might be instructive to contrast experimental study designs with the observational studies in humans that constitute the bulk of epidemiologic research. To select a nonrandom example, if one wished to test the hypothesis that cigarette smoking causes lung cancer in humans, an experimenter motivated only by scientific considerations would select sets of monozygotic twins (while lamenting from the point of view of the study design the absence of purebred genetic strains in this species), raise them in identical but separate cages, feed them identical diets and in general control other variables by the usual methods of experimental investigations. In addition, one member of each set, selected by appropriate means such as randomization, would be forced to smoke cigarettes regardless of any reluctance of that experimental subject to do so, and the other member of the set would be forcibly restrained from ever smoking cigarettes regardless of how much desire that experimental subject expressed to do so. Our hypothetical experimenter would continue this protocol and allow enough time (such as several decades) to elapse to determine whether the twins who smoked had substantially more lung cancer than the twins who did not.

In this hypothetical scenario there are numerous ethical and practical reasons why the scenario will remain hypothetical. Therefore, when this hypothesis was first seriously considered, investigators decided to take advantage of the fact that some members of the species, called smokers in our society, voluntarily exposed themselves to inhaled cigarette smoke and other members of this species conventionally called nonsmokers, voluntarily refrained from cigarette smoking.

There are two basic approaches that could be used in observational studies. The one I'll mention first is closer to the experimental situation; in this approach smokers and nonsmokers are observed to see whether the former appear to have a higher probability of developing lung cancer. In the second approach individuals with lung cancer and those free of the disease are asked for a history of their cigarette smoking habits.



However, in carrying out either of these investigations one would find that smokers differ from nonsmokers in many other characteristics besides cigarette smoking. For example, the age distribution is different. There are practically no smokers in individuals under the age of 6. Moreover, the proportion (or as the epidemiologist would say, the prevalence) of cigarette smokers differs considerably for different age groups. Smokers also differ from nonsmokers in other characteristics, for example, beverage consumption. Smokers are more likely to be coffee drinkers while nonsmokers are more likely to be tea drinkers. Unlike an experimental study, where the experimenter can control for these variables by such techniques as randomization and blocking, the investigator cannot automatically assume that cigarette smoking causes lung cancer if the results show that the risk of lung cancer is higher in smokers. In an observational study of one of the types described, such findings could be a result of a common underlying variable such as the known age differences between smokers and nonsmokers. More specifically, if in reality coffee drinking were etiologically related to lung cancer and cigarette smoking were not, an increased risk of lung cancer in those with a history of cigarette smoking would be found in such a study merely because coffee drinkers are more likely to smoke, unless methods are developed to be able to examine the effect of cigarette smoking in the absence of the effects of coffee drinking or with the effect of coffee drinking somehow taken into account. It would not be much of an exaggeration to say that the methods to do just that are the basis of epidemiology.

The specific study I'll be presenting today illustrates some of the practical difficulties encountered in epidemiologic studies. For this reason it may be a particularly appropriate choice for this program.

Perchloroethylene when administered by gavage was found to be associated with a significantly increased incidence of hepatocellular carcinoma in one of the two species tested. After these results became known, concern naturally arose that this chemical might be a carcinogen in man as well. This concern had considerable practical importance because perchloroethylene since its introduction in the late 1930's has gradually become used as a solvent more and more extensively in the dry cleaning industry.

This study was carried out by SRI International under contract with NIOSH, and the Project Officer, David Brown, was co-investigator. The study design involved the identification of individuals exposed to perchloroethylene and the comparison of their risk of death from specific causes with particular attention to cancer of specific sites with the risk in the general U. S. population of similar demographic characteristics. This kind of comparison is feasible, because information on the risk of death from specific causes has been routinely available for the U. S. population by age, sex, and race since 1933. Obviously, in order to carry out such comparisons the study population had to be large enough to allow these comparisons, the exposure to perchloroethylene had to occur over a long enough period of time so that health effects if any from that exposure would be likely to be induced, and a long enough period had to elapse after onset of occupational exposure to perchloroethylene for any health effects caused by that exposure to have enough time to occur. Therefore, minimum criteria to meet each of these requirements were written into the contract.

We had great difficulty in finding a population to study. This was an unexpected development, because the number of potential sources was large, and the sources were varied. However, with one exception, records identifying individuals exposed to perchloroethylene as a solvent in the dry cleaning industry were not retained by any of these sources for a long enough period of time to allow the study to be carried out. That exception comprised several unions of dry cleaners that could identify their members by the dry cleaning establishment where the member was employed and could supply the necessary demographic and other information to enable us to determine the fate of these individuals. Four large union locals with this information were the source of the study population.

If our mandate under the contract had been to study the risk of death from specific causes in dry cleaners workers, our problems in identifying the study population would have been over once we obtained the cooperation of these unions. However, the study population was intended to comprise dry cleaners workers exposed to perchloroethylene, and this meant only those union members who worked in shops where perchloroethylene was used as a solvent and who otherwise met the criteria for inclusion were eligible. Therefore, to determine which of the present and former union members were eligible, we had to identify those dry cleaning establishments which used perchloroethylene as a solvent and the dates that that chemical was so used, a task made even more difficult by the finding that none of these union locals had a master list of present and former member shops. After we laboriously compiled such lists from the union records, we found the majority of member shops were either closed or had changed ownership in the last few years. In the latter situation, the present owner never knew which solvent the former owner used and only rarely was the former owner available to provide us with this information. Therefore, we sought out other sources of information on solvent use including:

- Lists of purchasers of dry cleaning equipment using perchloroethylene provided by two cooperating equipment manufacturers;
- Union officials;
- Local distributors of dry cleaning equipment;
- Local distributors of perchloroethylene;
- Heads of trade associations covering these areas;
- Licensing authorities;
- Other individuals with a long knowledge of the local dry cleaning industry.

Even after exhausting all the sources that were willing and able to cooperate, more than half the dry cleaning establishments in these union locals had their solvent use unidentified. The study population was confined to those otherwise eligible who worked in establishments known to use perchloroethylene. It is obvious if solvent use information had been known for all member shops, the study population would have been considerably larger.

The fate of these individuals, whether alive, dead or unknown, was traced through the following sources by SRI: union records, the Social Security Administration, driver's license and motor vehicle registration information, the U. S. Post Office, and through the Internal Revenue Service by NIOSH. For those identified as deceased, death certificates were obtained from the vital statistics offices of the states in which death occurred. Cause of death was identified by a trained expert in the classification of disease and coded according to the International Classification of Diseases (a standard disease classification). Other information was coded, keypunched, and edited in a standard manner.

The results presented today are based on a study population of 1,597 eligible individuals--571 males and 1,026 females. As of September 30, 1977, 1,058 of these people were alive, 285 were dead, and 254 were of unknown vital status. The latter proportion is as high as it is because of the high proportion of females who are more difficult to follow because of changes in their last names and because they were less likely to be ascertained by the Social Security Administration and the Internal Revenue Service. Death certificates were obtained for all but 38 of the 285 individuals known to be deceased. Those deaths for which no certificates were found were assumed to have the same distribution by cause as those for which death certificates were available. Those who died after September 30, 1977 were treated as alive in the analysis.

Observed deaths by cause were compared with those expected from the comparable U. S. population. Each individual in the study population came under observation only after he or she had been employed one entire year in dry cleaning shops that were known to use perchloroethylene as a solvent during that time of employment. Observation was continued until September 30, 1977, the date of death of the individual, or the date at which the individual was lost to followup, whichever came first. Expected deaths were calculated based on the mortality experience of whites and of blacks separately. Unfortunately, in our study population we only knew the race of deceased individuals from their death certificates. If those who were deceased can be taken as representative of the entire study population, then approximately one-third of our study population was black.

In the results to be presented today, the expected deaths are calculated on the basis of a weighted average of those based on the mortality experience of whites and those based on the experience of blacks, with the weighting taking account of the proportions in each race by age over time among those study population members (namely, the deceased ones) for whom race was known. We have also calculated the results for expected deaths based on the assumption that all the population was white and those based on another assumption that all the population was black. These latter results are not presented today but are available upon request. For some causes of death, the results vary considerably, depending upon which race assumption is made, but for the causes of death of greatest interest, the differences based upon the different assumptions are rather small.

Because of time limitations, I will mostly discuss the results for cancer of the intestine except the rectum. All 11 deaths were actually from cancer of the large intestine. Some of the other causes with high ratios are based on very few observed deaths. Because of the small numbers they cannot be examined in

much detail and for the same reason are more likely to be chance findings. Cancer of the respiratory system is based on 19 observed deaths. However, the increased risk seen in cancer of the respiratory system is only found in males, while for females there are actually fewer observed deaths from that cause than would be expected on the basis of the mortality experience of the comparable U. S. female population. For this reason I will not discuss cancer of the respiratory system further in this talk.

I will also briefly mention the most remarkable finding of the study, the low risk in the study population of deaths from accidents. There was only 1 death from accidents and none at all from motor vehicle accidents. Perhaps this is a population that drives very little. These are relatively poor people who may not be able to afford automobiles and they also reside in large cities, with for the most part well developed public transportation systems and in many respects parking problems.

There are several reasons why cancer of the large intestine is discussed in more detail, which have to do with the general procedures for interpreting an epidemiologic study. These points include:

- The high ratio of observed to expected deaths from this cause based on 11 deaths. As I mentioned in my earlier discussion, in an epidemiologic study confounding variables cannot be controlled by randomization, blocking, or other techniques used in experimental studies. However, the higher this ratio, the less likely is there to be a confounding variable that could explain the association because the association with this hypothetical confounding variable would have to be at least as strong.
- The consistency of the association. The increased risk for this cause of death is seen in both males and females (in contrast to cancer of the respiratory system) and is also seen in each of the four union locals that participated in the study.
- Other possible explanations seem unlikely. For example, cancer of the large intestine is often confused clinically on death certificates with cancer of the rectum, so that sometimes an excess of deaths from one is entirely counterbalanced by a corresponding deficit of deaths from the other. However, there is an excess of observed to expected deaths for cancer of the rectum, although it is based on only 3 deaths. Also, it is known that those individuals of relatively high socioeconomic status are at the highest risk of cancer of the large intestine. Because of the working conditions of the dry cleaning industry, we believe that the study population tends to be primarily one of relatively low socioeconomic status, an otherwise low risk population for this cause. Because the expected number of deaths is generated from the general population's mortality experience--that is, a mixture of high and low risk groups--the ratio of observed to expected deaths may be underestimated.

OBSERVED DEATHS AND RATIO OF OBSERVED TO EXPECTED DEATHS  
 DRY CLEANERS EXPOSED TO PERCHLOROETHYLENE

	<u>Observed Deaths</u>	<u>Ratio of Observed to Expected Deaths</u>
Cancer of intestine (Except rectum)	11	1.82
Cancer of rectum	3	1.58
Cancer of the pancreas	5	1.52
Cancer of respiratory system	19	1.40
Cancer of urinary organs	5	1.98
Diseases of the blood and blood-forming tissue	3	2.90
Cirrhosis of the liver	5	0.67
Accidents	1	0.07
All deaths	285	0.93
All cancers	73	1.12

Ordinarily in this type of study one also examines the pattern of observed and expected deaths with total number of years of exposure to in this case perchloroethylene, and similar patterns for intervals since initial exposure to perchloroethylene. The first is roughly analogous with the dose/response relationship, which is important in experimental studies. In general, the longer one works the greater the risk would be if the risk is causally related to that particular exposure and if one takes into account (as was done in this study) the fact that individuals with longer duration of exposure tend to be older than those with shorter duration. Similarly, in such a situation one would expect that an occupationally related risk would show a pattern of being higher in those individuals for whom enough time had elapsed since they began to work for any occupationally related health effect to occur.

Unfortunately, in this particular study such examinations are less useful than usual. This is because we were unable to identify for many dry cleaning establishments whether or not perchloroethylene was used, which means that the total duration of exposure and the interval since first exposure, regardless of duration, will be underestimated for many individuals. Because it is not known whether this underestimation is more likely to occur for those individuals in the study who died from cancer of the large intestine, it is difficult if not impossible to interpret patterns with duration of exposure or interval since first known exposure to perchloroethylene.

Particularly because of the small numbers, it appears unwise to make definitive conclusions about specific causes of death from this study. However, the cause of death of greatest concern, because of the considerations discussed a few minutes ago, is cancer of the large intestine. Because the small numbers preclude definitive conclusions, we have recommended in our report that the study be repeated in several years to generate larger numbers, which would allow the best hope of establishing definitively whether there is a problem with cancer of the large intestine in this population. Another possible method of getting larger numbers would be to expand the number of individuals in the study population. However, because of the unlikelihood of getting any further information on solvent use to identify additional individuals in the union membership who would qualify for the study population, enlarging the number of individuals in the study population (as opposed to the time that the same numbers of individuals are followed) appears impractical. If the study is repeated, it is also recommended that special attention be paid to other causes of death that show an increased ratio of observed to expected deaths but have relatively small numbers of deaths from these causes by September 30, 1977.

THE MORTALITY EXPERIENCE OF ARMY WORLD WAR II  
CHEMICAL PROCESSING COMPANIES

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INTRODUCTION

BACKGROUND

Occupational exposure to a wide variety of chemicals is known to contribute significantly to the risk of certain cancers, particularly of the lung, bladder, liver, and leukemia. Although many compounds have been shown to produce cancer in laboratory animals, for only about 20 or 30 are the data in man considered convincing, usually on the basis of occupational studies (World Health Organization, 1972-1975; Cole and Goldman, 1975).

Experimental evidence has accumulated which indicates that chlorinated hydrocarbons are carcinogenic to laboratory animals. The liver is often involved, but cancers of the respiratory tract, kidney, and other sites have been reported. Exposures to chlorinated hydrocarbons in man result from industrial chemicals, pesticides, and in some instances, contaminated drinking water, but most exposures are recent and few epidemiologic studies of such exposed populations have been undertaken. Vinyl chloride is known to cause liver angiosarcoma in man and may be linked to cancer of the lung and brain following occupational exposure. Bichloromethyl ether has been linked to oat-cell carcinomas of the lung in exposed workers. A third member of this class of compounds, 1,1,2,2-tetrachloroethane or, as it is alternatively named, acetylene tetrachloride, which is known to be toxic to the liver in man, has been widely employed as a solvent for cellulose acetate in airplane "dope", in fire extinguishers, and in the manufacture of artificial silk, synthetic fibers, and insecticides, but has yet to be implicated as carcinogenic by any study of an exposed human population.

## ARMY CHEMICAL PROCESSING COMPANIES

Tetrachloroethane was used by the Army during World War II in the impregnation of clothing against mustard gas and this paper presents the results of a study of mortality following that occupational exposure. The Chemical Processing Company was a basic unit in defense against gas warfare (Kleber and Birdsell, 1966). Its primary mission was to keep available to theater chemical officers a supply of permeable protective clothing adequately and recently impregnated with chlorinating compounds that would protect the wearer from the effects of vesicant vapor or droplets. A company consisted of 146 men, organized in two platoons. Each platoon was the organizational equivalent of a processing plant and contained three functional sections for continuous three-shift operation.

Each company was supplied with two plants designed for impregnating clothing with the protective compound. Two types of plants were developed and used during the course of the war. The original impregnation process was accomplished using the M-1 plant which required the use of tetrachloroethane as a solvent for the impregnate, *N, N*-dichloro-hexachloro-diphenyl-urea, code named CC-2. Tetrachloroethane is highly toxic; prolonged exposure to its fumes produced weakness and nausea associated with hepatic stress requiring immediate relief from chemical processing duty. Furthermore, the corrosive nature of the solvent and the necessity of its recovery for re-use produced maintenance problems and recurrent mechanical malfunctions. The Army Chemical Warfare Service therefore placed a requirement for a water soluble impregnate. Technicians met this need by reducing the individual particle size in CC-2 to micron proportions and designing an M-2 plant which used the water suspension method. These plants were introduced in mid-1943 and deployed in both the European and Pacific theaters.

Thirty-nine Chemical Processing Companies were formed during the war and all but one of them served overseas (Brophy et al., 1959). In the absence of gas warfare, the companies were given secondary missions of providing dry cleaning and laundry services to field troops. In the tropics, the plants were also used to impregnate clothing with insecticides as a preventive for scrub typhus and malaria.

An examination of the unit histories and other records of the Chemical Warfare Service revealed that ten companies used the M-1 plant with the tetrachloroethane solvent process and the remaining companies were supplied with M-2 plants.

## MATERIALS AND METHODS

### DESCRIPTION OF THE SAMPLE

The 13,333 men who served in the 39 Chemical Processing Companies during the active duty period of World War II comprise the gross study sample. It was assembled from unit records (morning reports, payroll rosters, and personnel rosters), generally in the form of microfilm files. Items abstracted were name, military service number, company number, date joined, rank at time of joining, duty assignment (a three-digit code for the military occupational specialty or M.O.S.) and date, the date of transfer, rank at time of transfer, and the type



of transfer, whether to another processing company, to another type Chemical Warfare Service unit, to detachment of patients, or other. Years of birth were obtained from a microfilm copy of a punchcard prepared for each serviceman at the time of his induction. There were three all black companies and the remaining units were white, so that separate racial comparisons were made simply on the basis of company assignment.

#### DEFINITION OF COMPARISON GROUPS

This study was undertaken for the purpose of learning whether exposure to tetrachloroethane, either by direct skin contact or through inhalation of its vapor, was the beginning of a carcinogenic process which would manifest as excess cancer mortality over a 30-year follow-up period. However, the secondary mission of these companies meant that the same men directly involved in the impregnation of clothing were also exposed, in some degree, to the solvents used in dry cleaning. It was not possible to document exposure to any of the solvents on a man-for-man basis because a single set of military occupational specialty codes (M.O.S.) was assigned for duties involving either impregnation, laundering, or dry cleaning. Accordingly, all men with M.O.S. codes in this category were combined into one group designated "P,L,D" for processing, laundry or dry cleaning duties. The remaining codes were labelled "other". For nearly a quarter of the entire study sample no specialty code was found. Those men who served in one of the ten companies which used the M-1 plants requiring tetrachloroethane as a solvent and whose M.O.S. fell in the "P,L,D" category thus comprised, a priori, the primary case group. Men with service limited to the remaining companies and a "P,L,D" category M.O.S. form a logical secondary group of cases whose exposure was restricted to dry cleaning solvents. In the analysis, comparisons of mortality have been made for these two groups both separately and combined.

#### MORTALITY ASCERTAINMENT

The fact and date of death was obtained from the Veterans Administration Beneficiary Identification and Records Locator Subsystem (BIRLS), a computerized index to veteran contacts with that organization which requires name and service number as input. The mortality of almost all war veterans, regardless of any prior association with the VA, is a matter of record in VA files. The certified cause of death in each instance was obtained from the application for death benefits, which includes a copy of the death certificate. From past investigations it has been estimated that mortality information is retrievable for about 95 percent of World War II veterans (Beebe and Simon, 1969).

Upon completion of mortality ascertainment through 31 December 1976, 76 men were found to have died prior to 1 January 1946, the beginning of the follow-up period, and two others died at an unknown date. After eliminating these 78, the study sample numbered 13,255 men, 12,297 white and 958 black males. Deaths among whites numbered 2,414 and among blacks 240 during the follow-up period, or 19.6 percent and 25.0 percent, respectively.

Causes of death were coded according to the Eighth Revision of the International Statistical Classification of Diseases, Injuries and Causes of Death of the World Health Organization at the four-digit level. Most comparisons made in the analysis use expected number of deaths for the follow-up period. These values were obtained by application of cause, age, race, and calendar-year-specific U. S. mortality rates to the surviving cohorts of successive follow-up years. Confidence limits on relative risks have been calculated using the method of Woolf and Haldane (1955-56).

## RESULTS

Gross mortality by race, type of solvent used, and military occupational specialty is shown in Table 1. The 2,414 deaths among white males represent 92.8 percent of expectation based on age and calendar-year-specific U. S. mortality rates for the 31-year follow-up period. This deficit characterizes most mortality studies of World War II servicemen, reflecting the persistent effect of screening provided by the military induction process. The 240 deaths among blacks is 68.3 percent of expectation for U. S. non-white males for whom entry into military service is a more highly selective mechanism than for whites. Only 163 deaths from all disease were recorded among those blacks for whom an M.O.S. was available, and of these there were just 42 deaths ascribed to malignancies, 8.0 in the P,L,D, group as compared to an expected 12.5 deaths. There were 34 cancer deaths among blacks with other known M.O.S.'s, as compared to 34.9 expected. Because the small number of blacks affords mortality comparisons of negligible power, the remaining analysis is restricted to whites.

TABLE 1. COHORTS BY RACE, TYPE OF SOLVENT USED, AND  
PRINCIPAL MILITARY OCCUPATIONAL SPECIALTY

Type of process	M.O.S.	Whites		Blacks	
		Number in cohort	Deaths 1946-76	Number in cohort	Deaths 1946-76
Tetrachloro- ethane solvent	P,L,D	1,099	194	124	23
	Other	1,319	277	393	110
	Unknown	827	202	97	27
Water solvent	P,L,D	3,166	576	101	24
	Other	3,924	744	141	39
	Unknown	1,962	421	102	17
Totals		12,297	2,414	958	240

The 194, 277, and 202 deaths among the P,L,D, Other, and Unknown M.O.S. groups in companies using tetrachloroethane represent 86 percent, 92 percent, and 81 percent, respectively, of the corresponding expected mortality in each group. Similarly, the 576, 744, and 421 deaths among the M.O.S. groups of the companies using the water solvent process are 96 percent, 94 percent, and 95 percent, respectively, of expected mortality. Before examining the cause-specific mortality

for each of these groups, a look at gross mortality by military rank is necessary, since Keehn (1978) and Jablon et al. (1977) have found a significant relationship between the two. Table 2 shows that privates and privates first class have overall identical mortality ratios of 95 percent, while the 287 deaths among corporals and sergeants is 88 percent of expectation. These figures are in basic agreement with previous findings, but the magnitude of the effect is not sufficiently large to warrant subsequent rank specific analysis of mortality by cause. At the level of M.O.S. within rank, only the 96 deaths (13 percent of all P,L,D deaths) among corporals and sergeants with duties in the P,L,D category represent a mortality ratio significantly less than 100 percent ( $P = .028$ ).

TABLE 2. TOTAL MORTALITY AMONG WHITES BY PRINCIPAL M.O.S. AND RANK, 1946-76

Military rank	M.O.S.	Observed deaths	Expected deaths*	Mortality ratio (M.R.)	Overall M.R.
Privates	P,L,D	497	528.18	.94	.95
	Other	622	653.05	.95	
Privates First Class	P,L,D	152	144.65	1.05	.95
	Other	168	190.94	.88	
Corporals & Sergeants	P,L,D	96	120.13	.80	.88
	Other	191	207.85	.92	
Unknown	Unknown	623	690.05		.90

\*Calculated by a life-table method using U. S. mortality rates for white males, specific to age and calendar year, applied to corresponding cohorts.

Mortality from all diseases, all malignancies, cardiovascular disease, and liver cirrhosis by solvent used and principal M.O.S. is presented in Table 3. Mortality ratios for all diseases are remarkably close for all M.O.S. groups who served in companies which used the water solvent process. Among the companies using tetrachloroethane, the three corresponding mortality ratios are more dispersed but do not differ significantly. In both instances, those men with unknown M.O.S.'s have the lowest mortality. This emphasizes the presence of chance variation in a situation where logically, if the P,L,D M.O.S. exercised a real effect on total mortality, these ratios would tend to fall between those of the two groups with known M.O.S. As the largest component of total mortality, deaths from cardiovascular disease follow the same pattern across solvent type and M.O.S. categories. Here, also, the mortality ratios of .79, .94, and .79 of the tetrachloroethane plant cohorts do not differ significantly. Deaths from liver cirrhosis are notably lower in both P,L,D groups, significantly so among the larger water solvent process cohorts where the relative risk is .49 with 90-percent confidence limits of .31 and .81. This effect may be due to the fact, mentioned in two unit histories, that inhaled fumes of tetrachloroethane or dry cleaning solvents produced hepatic stress requiring relief from P,L,D duty for

habitual moderate to heavy drinkers. It is possible that these persons were given other assignments because of this phenomenon.

TABLE 3. OBSERVED MORTALITY AND STANDARD MORTALITY RATIOS FOR MAJOR CAUSE GROUPS BY SOLVENT USED AND PRINCIPAL M.O.S.

Type of process	Principal M.O.S.	All diseases	All malignancies	Cardiovascular disease	Cirrhosis of the liver
Tetrachloro-ethane solvent	P,L,D	161 (.84)	41 (.96) } <sup>a</sup>	82 (.79)	4 (.48) } <sup>c</sup>
	Other	233 (.89)	44 (.76) }	135 (.94)	10 (.97) }
	Unknown	177 (.79)	35 (.72)	99 (.79)	13(1.81)
Water solvent	P,L,D	475 (.94)	117(1.04) } <sup>b</sup>	249 (.92)	15 (.67) } <sup>d</sup>
	Other	629 (.93)	138 (.92) }	320 (.88)	39(1.36) }
	Unknown	354 (.92)	73 (.86)	185 (.88)	23(1.52)

a: RR = 1.26; 90% C.L. = (.89, 1.80)

b: RR = 1.13; 90% C.L. = (.91, 1.38)

c: RR = .49; 90% C.L. = (.22, 1.30)

d: RR = .49; 90% C.L. = (.31, .81)

Cancer mortality ratios are greatest in the P,L,D M.O.S. cohorts for both types of solvents, although neither of the risks relative to the other M.O.S. cohorts, 1.26 for tetrachloroethane and 1.13 for water, differ significantly from one. Observed and expected mortality by site of malignancy, M.O.S., and solvent type is displayed in Table 4. The largest relative risks occur for tumors of the genital organs (4.56, with a 90-percent confidence interval of .85 to 14.75), leukemias (1.77, with a 90-percent confidence interval of .57 to 5.14), and other lymphatic cancers (5.19, with a 90-percent confidence interval of .98 to 15.4) within the cohort with tetrachloroethane exposure. Three of the four deaths from other lymphatic cancers were lymphosarcomas, for which the expected mortality is 0.85. This yields a chi-square with a P value of 0.02. However, this result must be placed in perspective by observing that there were, in this cohort, deaths from 23 different types of cancer and the probability of finding at least one with a P value of .02 or smaller by chance alone is 0.37. For the two cohorts which used the water solvent, none of the cancers have relative risks significantly greater than one, gauged by 90-percent confidence intervals. The values of Table 4 have been used to calculate two sets of relative risks which appear together with 90-percent confidence limits in Table 5. When only those persons with P,L,D assignments are considered, the relative risk of the tetrachloroethane vs. water cohorts for all malignancies is just 0.92 with 90-percent confidence limits that include one. None of the sites show significant elevations in risk; tumors of the genital organs, leukemias, and other lymphatic cancers, as in the previous internal comparison, are the only types with relative risks larger than one. Only leukemia, with a relative risk of 2.72 and 90-percent confidence interval of .96 to 7.70, is suggestive. In the second half of Table 5 the risks of the P,L,D cohorts relative to those with other known M.O.S.'s are seen to be greater than one for all sites combined as well as for each individual site, even though none except the value for total malignancies are suggestively large. Figure 1 provides a graphic comparison of cancer mortality over time for these two M.O.S. cohorts. Cancer survival per 1,000 men at risk is plotted against

calendar year of follow-up. In these curves, persons dying of causes other than cancer have been treated as withdrawals from observation in the year of their death. Although the Mantel chi-square test (1966) for comparing these two curves showed no significance ( $P > .05$ ), the slight excess overall cancer mortality among the P,L,D cohort can be seen to have started after 1966 and continued through 1976, the last year of follow-up.

TABLE 4. OBSERVED AND EXPECTED CANCER MORTALITY  
BY MAJOR SITES FOR WHITES BY SOLVENT USED AND  
PRINCIPAL M.O.S.

Site or type of malignancy	Tetrachloroethane		Water	
	P,L,D	Other	P,L,D	Other
Buccal Cavity	1 (1.59)	2 (2.12)	4 (4.16)	4 (5.47)
Digestive Organs	9(11.18)	10(15.54)	24(29.17)	29(39.12)
Respiratory Tract	13(15.35)	19(20.73)	49(40.40)	57(53.25)
Genital Organs	3 (1.65)	1 (2.51)	5 (4.24)	8 (5.96)
Urinary Organs	0 (2.09)	1 (2.94)	4 (5.47)	3 (7.35)
Leukemia & Aleukemia	4 (1.81)	3 (2.40)	4 (4.92)	6 (6.41)
Other Lymphatic	4 (3.07)	1 (3.98)	8 (8.35)	8(10.78)
Other Malignancies	7 (6.08)	7 (7.92)	19(16.37)	23(21.23)

TABLE 5. RELATIVE RISKS AND 90% CONFIDENCE LIMITS FOR  
CANCER MORTALITY BY MAJOR SITES FOR WHITES

	Tetrachloroethane vs. Water for P,L,D M.O.S. only		P,L,D M.O.S. vs. Other M.O.S. for both solvents	
	R.R.	90% C.L.	R.R.	90% C.L.
All Malignancies	.92	(.69, 1.25)	1.16	(.97, 1.38)
Buccal Cavity	.66	(.22, 3.47)	1.09	(.44, 2.77)
Digestive Organs	.98	(.55, 1.87)	1.15	(.78, 1.69)
Respiratory Tract	.70	(.44, 1.18)	1.08	(.82, 1.43)
Genital Organs	1.58	(.58, 4.83)	1.28	(.61, 2.74)
Urinary Organs	0.00	(0, 1.76)	1.36	(.48, 3.84)
Leukemia & Aleukemia	2.72	(.96, 7.70)	1.17	(.55, 2.50)
Other Lymphatic	1.35	(.57, 3.59)	1.73	(.86, 3.41)
Malignant Other	.99	(.52, 2.06)	1.13	(.73, 1.75)

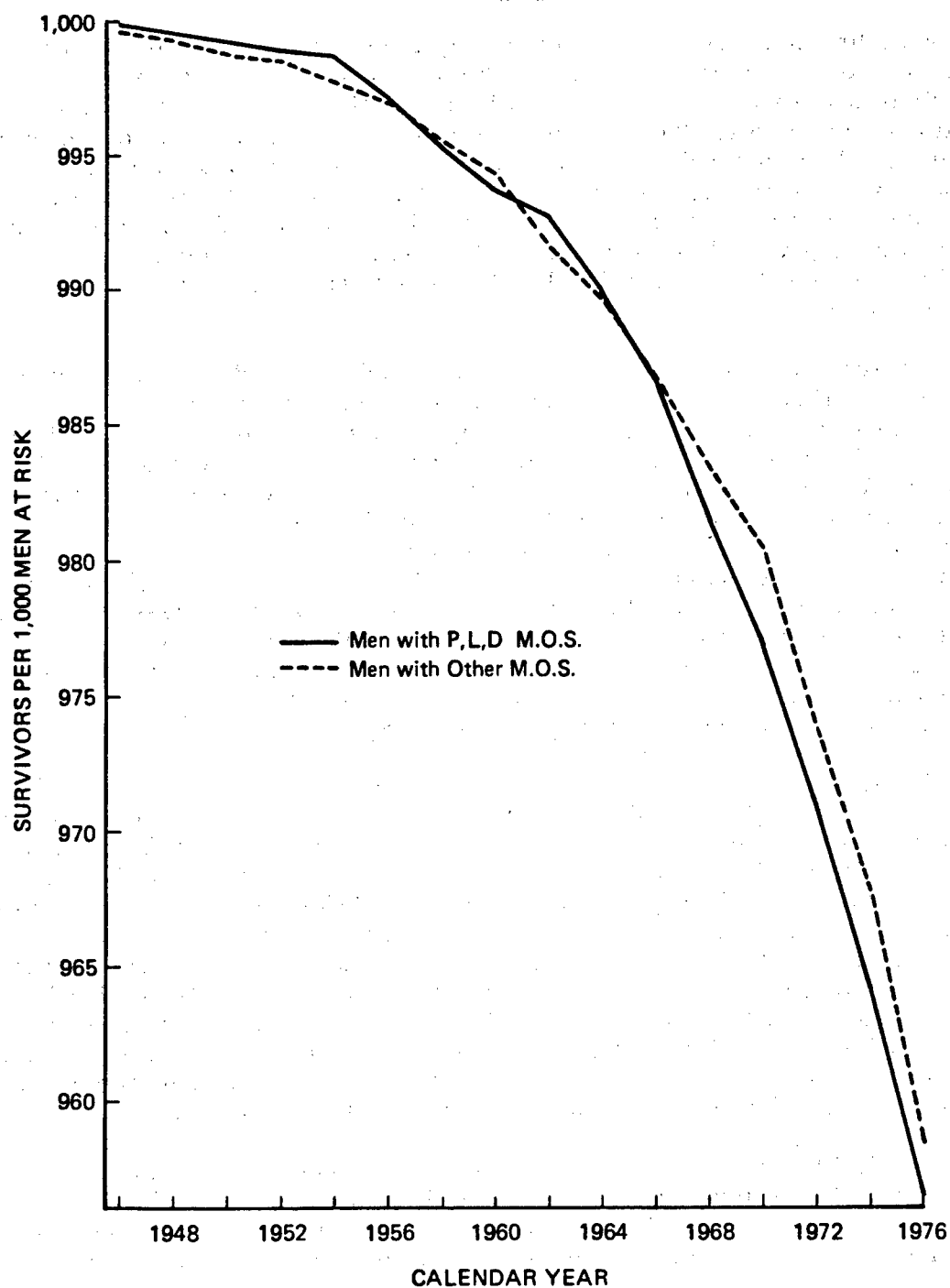


Figure 1. Cancer survival curves for chemical processing, laundry, and dry cleaning M.O.S. (P,L,D) and for all other known M.O.S.

## DISCUSSION

The possibility that tetrachloroethane might be carcinogenic in man has been suggested by two observations. It is similar in molecular structure to several other chlorinated hydrocarbons known to increase the risk of certain cancers in occupational exposures. These include vinyl chloride (liver angiosarcoma), bichloromethyl ether (oat-cell lung carcinoma), and tetrachloroethylene, a solvent used in dry cleaning (Blair et al., 1979). Further, bioassay studies of both tetrachloroethane and tetrachloroethylene have produced hepatocellular carcinomas in mice given these substances by gavage (U. S. Department of Health, Education and Welfare, 1977; U. S. Department of Health, Education and Welfare, 1978).

In this study, 1,099 white males were identified who had some exposure to tetrachloroethane through work assignments requiring proximity to the machinery which used the solvent to impregnate clothing with a substance to neutralize mustard gas. Their overall cancer mortality for the 31-year period 1946-76 is 1.26 times that of the 1,319 men who served in the same companies but with duties which did not involve them in the impregnation process. This excess is due primarily to moderately elevated risks for tumors of the genital organs, the leukemias, and other lymphatic cancers. The corresponding P,L,D cohort within the group of companies which used the water solvent process has a 1.13 relative risk for all cancer mortality, with very slight excesses for all major types, though none beyond the limits of chance. The largest of these is for other lymphatic cancers, where the contribution is chiefly due to the excess risk in the P,L,D cohort with tetrachloroethane exposure.

The unit histories for seven companies which used tetrachloroethane indicate that chemical processing with this solvent was done for very limited periods of time ranging from five weeks to a year, with an average of about five months. Since gas warfare was never employed in World War II, re-impregnation of uniforms was required at intervals governed by deterioration of the impregnate due to routine use and exposure to heat and moisture. The absence of a pronounced excess of deaths from any form of malignancy underlines the very limited exposure of this study cohort. Li et al. (1969) found excess deaths from both leukemia and lymphatic cancers in a 20-year follow-up of members of the American Chemical Society. Thus, the moderately increased relative risks for deaths from cancers of these two sites among the tetrachloroethane/P,L,D cohort may in fact be linked to wartime exposure to this substance.

The very slight excess risk for all malignancies among the combined P,L,D cohorts is difficult to interpret. A global significance level for this result, assuming chance deviations above and below unit relative risk to be one half, is 0.004. The subsequent occupation history of these men is not known and extensive post-war employment in the dry cleaning business seems unlikely, since this is an occupation dominated by unskilled minorities, notably women and non-whites. A comparison of the cancer mortality made for men in the P,L,D cohorts who entered service before July 1943 and those inducted after revealed no difference which could be ascribed to a greater exposure to either tetrachloroethane or dry cleaning solvents. If these men are experiencing heightened cancer mortality, those factors which led to their assignment to the

the Chemical Warfare Service with processing, laundry, and dry cleaning duties, rather than their wartime experience, must provide the rationale.

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## ACKNOWLEDGEMENT

This study was planned and initiated by Dr. C. Dennis Robinette of the Medical Follow-up Agency under the direction of Dr. Joseph F. Fraumeni, Jr., of the Environmental Epidemiology Branch of the National Cancer Institute. It was supported by the National Cancer Institute Contract No. NO1-CP-5-3573 as part of a series of investigations of cancer epidemiology.

The Medical Follow-up Agency conducts a program of studies developed by the Committee on Veterans Medical Problems and carried out in cooperation with the Veterans Administration, Department of Defense, and the National Institutes of Health. The program is currently conducted with the advice of the Committee on Epidemiology and Veterans Follow-up Studies. The use of Army records in this study is acknowledged but is not to be construed as implying official approval by the Department of the Army of the conclusions presented. The study depended on the records of the Department of Defense, the Veterans Administration, and the National Archives Records Service, GSA. The authors are indebted to these agencies for the ready access they provided to the required materials. Study rosters were assembled by the staff of Mrs. Dorothy J. Mahon, NRC representative in the Federal Records Center, St. Louis, Missouri, and causes of death were coded by Mrs. Vivian A. Farley and her staff. Miss Chiquita M. Hicks coordinated the assembly and editing of the data tape file and prepared all tables for the analysis.

PRELIMINARY RESULTS AND COMPARISON OF JAPANESE,  
FINNISH, AND UNITED STATES CARBON DISULFIDE  
MICROANEURYSM STUDIES

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INTRODUCTION

Retinal angiopathy was first associated with carbon disulfide when Goto and Hotta (1967) found increased incidence of retinal small hemorrhages or microaneurysms, nephropathy, anemia, and a variety of subjective symptoms among workers exposed to carbon disulfide in a rayon plant in Japan (Goto and Hotta, 1967; Goto et al., 1971; Goto et al., 1972). Additional studies in Japan and Yugoslavia supported these findings and demonstrated an abnormal response to corticosteroid-augmented glucose tolerance testing among workers exposed to carbon disulfide (Hernberg et al., 1970; Hotta et al., 1972)

Although there was no information on exposure level in these early reports, and they were often flawed by small comparison groups, there seemed no strong reason to doubt a connection between carbon disulfide exposure and a microangiopathy mediated by abnormal glucose metabolism until Raitta (1974) reported results of her examinations of 100 workers exposed to carbon disulfide in a Finnish rayon plant previously described by scientists at the Institute of Occupational Health in Helsinki. (See Figure 1.) Aneurysms were much less common in both the exposed and unexposed groups in Finland than in Japan; no effect of carbon disulfide was found on the prevalence of aneurysms; and there was no effect on blood sugar which could be detected by a standard glucose tolerance test not augmented with corticosteroids. Bias in selection of the Japanese cohort was considered the most likely explanation.

In 1976, Sugimoto, Goto and Hotta (1976) reported a "five-year follow-up on retinopathy due to carbon disulfide". They concluded that retinopathy progressed more in the group that remained exposed and that regression in grade of retinopathy occurred significantly more often in workers removed from carbon disulfide exposure than in those remaining in the same work site. While environmental levels again were not given, they recommended reduction in the Japanese TLV ( $60 \text{ mg/m}^3$ , or about 19 ppm) for carbon disulfide.

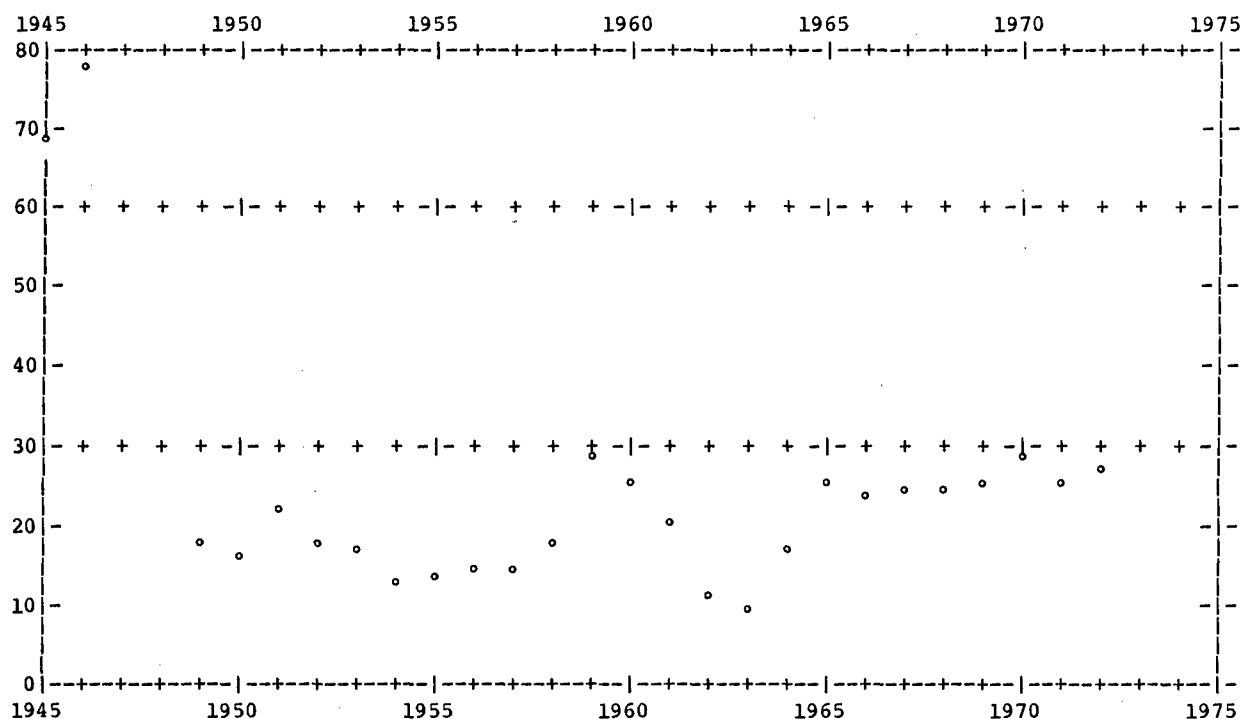


Figure 1. Environmental levels of  $\text{CS}_2$  in the Finnish rayon plant studied by Ch. Raitta, M. Tolonen, & M. Nurminen. 1974. (P. 153) Levels shown are approximately those of the spinning department, which had higher levels than the xanthation room and viscose ripening room, but lower than the spinning bath room.

By 1977, the Japanese and Finnish groups had combined forces and completed a collaborative study of workers in each country. Environmental levels in the Japanese plant, described in 1976 by Tolonen and others (see Figure 2), were in the same range as those in the Finnish plant (Sugimoto et al., 1977). The ophthalmologic report essentially confirmed the earlier findings that retinopathy was rare in Finland, more common in Japan, and associated with carbon disulfide exposure in Japan but not in Finland (Sugimoto et al., 1976).

About this time, planning was begun for NIOSH's cross-sectional medical and industrial hygiene study of workers exposed to low levels of carbon disulfide. With findings suggesting a problem in Japan and Yugoslavia but no problem in Finland, it seemed prudent to determine where U. S. workers fit in this scheme.

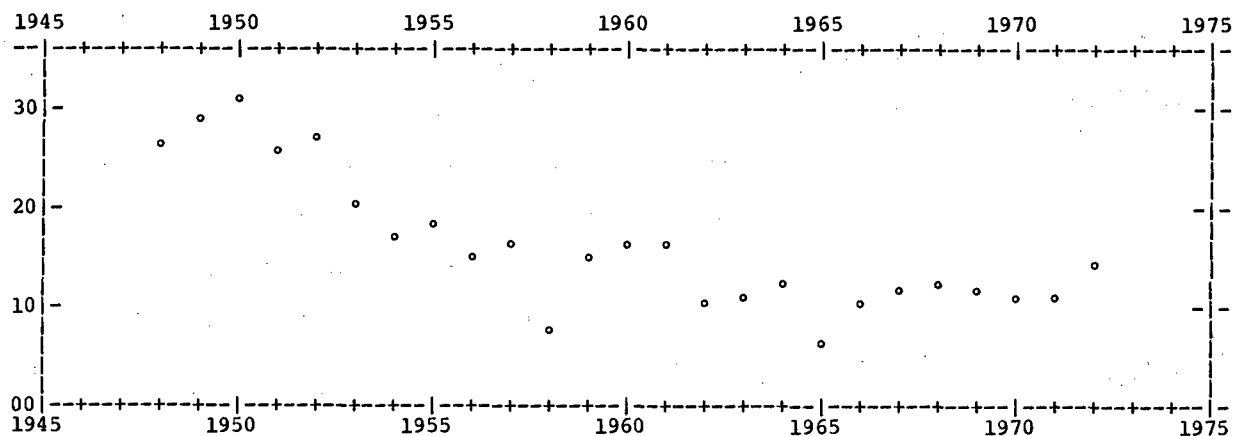


Figure 2. Environmental levels of CS<sub>2</sub> in the Japanese rayon plant studied by M. Tolonen, S. Hernberg, C-H. Nordman, S. Goto, K. Sugimoto, & T. Baba. 1976. (P. 253) Values above correspond approximately to the levels for "2nd room" as given in the paper. This room had, in general, the highest levels of the three, but is still not much different from the others.

## METHODS AND MATERIALS

### SELECTION OF A COHORT

After meetings with labor and industry representatives and visits to the four U. S. rayon staple plants, a factory in northeastern Tennessee was chosen as the study site. Rayon staple is a product like cotton wool, used in disposable diapers and sanitary napkins among other products. The plant was opened in 1948 for production of rayon filament (thread or yarn). A viscose rayon staple plant was opened in 1956; a nylon filament plant in 1963; a polyester filament plant in 1966; and a nylon-polyester staple plant in 1967. The rayon filament plant ceased production in 1974 (see Figure 3). The plant was considered among the best controlled in the United States, with environmental area samples going back to 1957 typically showing air concentrations of carbon disulfide between 1.5 ppm and 60 ppm. Levels are generally similar to those noted in Finland and Japan (see Figure 4).

The exposed group was chosen from currently employed members of the viscose rayon staple plant who had been employed in that plant for at least one year. Their employment prior to that time could have included the old rayon filament plant or one of the other synthetic plants listed. Two hundred seventy-three workers were potentially available who fit this description; 189, or 69%, of them signed informed consent forms.

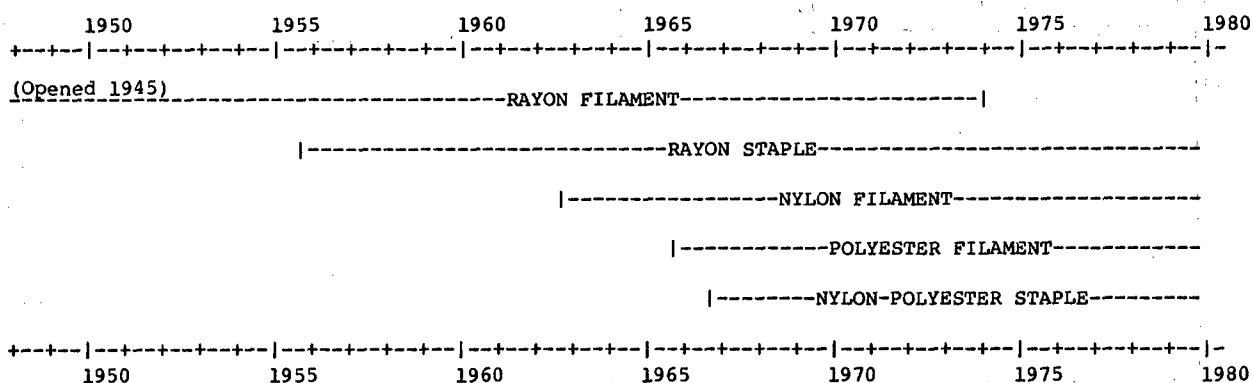


Figure 3. Chronology of plant additions at a site in northeastern Tennessee.

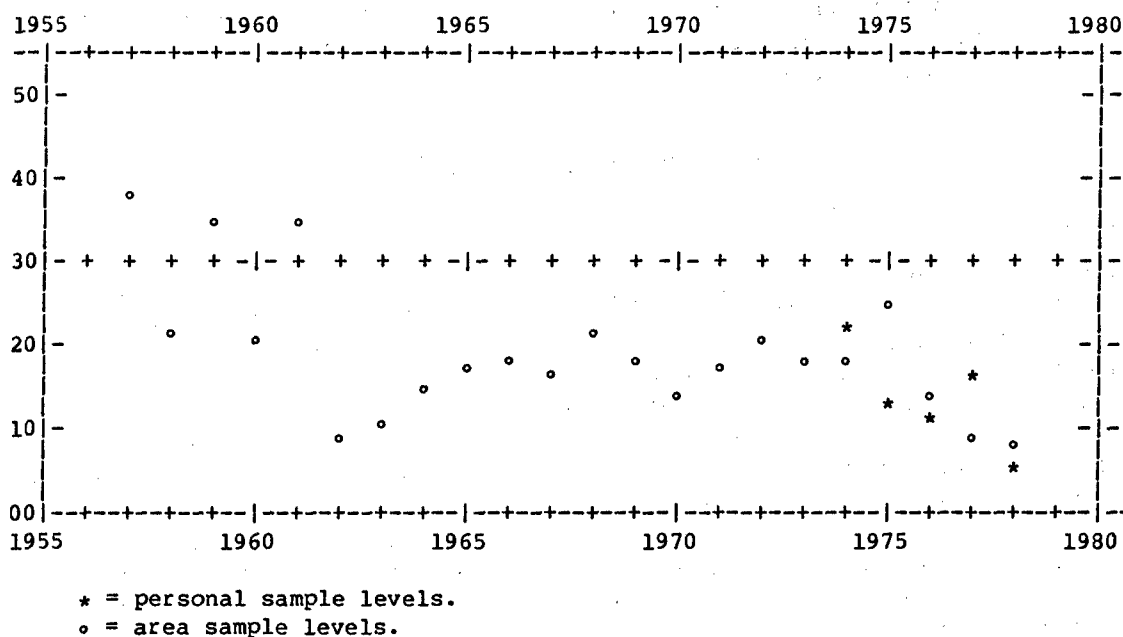


Figure 4. Levels of  $CS_2$  in a viscose rayon staple plant in northeastern Tennessee. NIOSH. 1979.

The control group was selected from either the polyester filament, nylon filament, or nylon-polyester staple plant, where there was no occupational exposure to carbon disulfide. Those included had to have been employed at least one year in one or more of these plants, but never employed in either the rayon staple or rayon filament plants. Four hundred twenty-two workers fit this description; 244, or 58%, signed informed consent forms.

Because the incidence and prevalence of cardiovascular disease differ between races, and because there were only twelve non-white employees in the exposed group, only white employees were used for the final analysis. Also lost to final analysis were twenty-one of the exposed workers for whom no exposure data were obtained. One hundred fifty-six exposed and 233 control subjects were used for the final analysis.

#### DEMOGRAPHIC COMPARABILITY OF EXPOSED AND COMPARISON GROUPS

General information including name, address, social security number, age, race, and educational level, occupational history (both at the study site and prior to employment at the study site) and smoking history was obtained by questionnaire (which is available on request). See Table 1.

TABLE 1. AGE AND EDUCATION LEVELS OF EXPOSED AND UNEXPOSED WORKERS

		Exposed	Unexposed
Age	M	38.24	33.86
	SD	10.11	9.04
Education	M	10.48	11.06
	SD	2.00	1.74

#### AGE

The average age of the exposed group was 38.2 and that of the comparison group 33.9. This difference was statistically significant using Student's t-test (p less than 0.01).

#### EDUCATION

The average number of years of education for the exposed population was 10.5 years and for the comparison 11.1 years. Using Student's t-test this difference was significant at p less than 0.01.

#### OCCUPATIONAL HISTORY

Occupational history revealed that there was no significant difference in previous occupational exposed to various potentially hazardous agents between the carbon disulfide exposed and the control groups.

#### SMOKING HISTORY

From the questionnaire results, subjects were grouped according to whether they were smokers, ex-smokers, or non-smokers. There was a significant difference between the exposed and control groups when cigarette smoking was considered by pack years. (p = 0.03)

## EXAMINATION PROCEDURES

Subjects were seen in a randomized order. No information on exposed history was on forms and examiners were prohibited from asking questions or engaging in conversation which would yield this information. Each subject underwent pupillary dilation with a short acting mydriatic. After dilation was complete, direct ophthalmoscopy was performed and the results recorded. Each subject then had two pictures taken of each retina using a Topcon retinal camera, a monochromatic light source, and panchromatic film. The film was later processed and mounted as black and white slides. Slides were identified only by a randomly-assigned code number and read by ophthalmologists.

## RESULTS

Eye examination results were initially reported as showing or not showing microaneurysms, as showing or not showing hemorrhages, and as showing or not showing other abnormalities. Both exposed and control groups were found to have about 25% prevalence of microaneurysms. Although our technique differed from those in the Finnish and Japanese reports cited earlier, the high prevalence of microaneurysms in the control group was unexpected, even if an association with carbon disulfide exposure exists. Since the concordance between the two independent readers was very good (see Table 2), it seemed possible that the population chosen for the study was, for some reason, unusual. Further inquiry and consultation with additional ophthalmologists revealed that the initial classification system had been too simple; experts advised that a large fraction of the slides could be expected to show small dots that were not clearly aneurysms but were also not clearly artifact. Figure 5 shows a normal retina. Figure 6 shows a retina with definite aneurysms. Figure 7 shows an example of a retina with a dot that is not as easily interpretable and is therefore not likely to be artifact.

TABLE 2. CONCORDANCE OF INITIAL TWO READERS OF  
RETINAL PHOTOGRAPHS

		Reader "M"		
		Microaneurysms		Total
		Present	Absent	
Reader "R "	Present	107	6	113
	Absent	12	304	316
	Total	119	310	429

Arrangements were then made to have the slides read by a new ophthalmologist, reporting results separately for each subject's right and left eye as either normal, having definite or uncertain microaneurysms, or having definite or uncertain hemorrhages.



Figure 5. Example of normal retina with no sign of aneurysms.



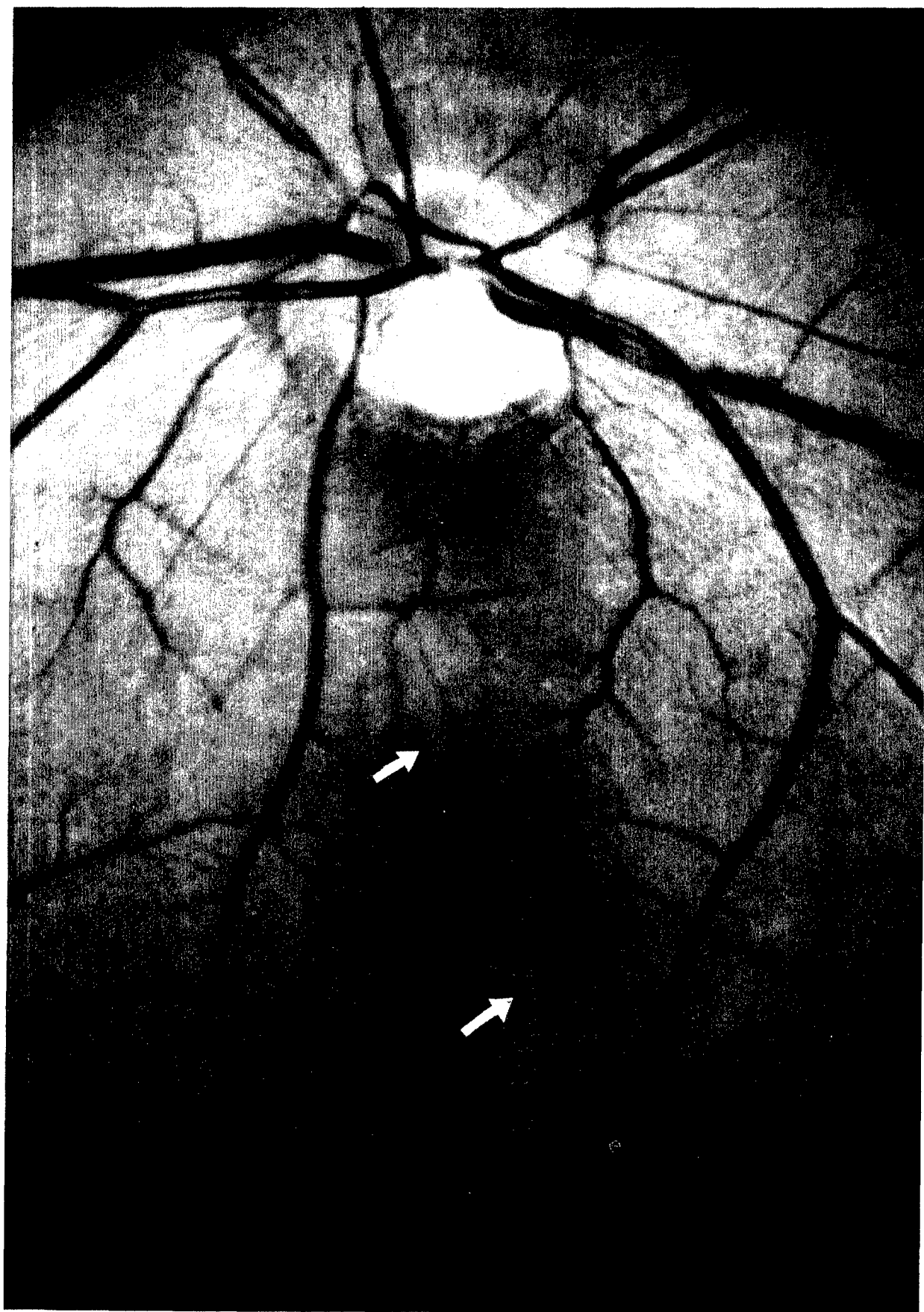


Figure 6. Example of a retina showing definite lesions (arrows).



Figure 7. Example of a retina showing signs of a possible aneurysm (arrow).

## RELATIONSHIP OF EYE FINDINGS TO CURRENT EXPOSURE

Table 3 shows results for the control versus exposed analysis, using the second grading method. The exposed group had almost 20 percent retinal microaneurysms (both definite and uncertain) compared to 7.5 percent for the control group (significant at  $p$  less than 0.01). The exposed group had 10.5 percent retinal hemorrhages (both definite and uncertain) compared to 3 percent for the control group (significant at  $p$  less than 0.01).

TABLE 3. RETINOPATHY (CONTROL VERSUS EXPOSED)

	Control		Exposed		Total	
	N	Percent	N	Percent	N	Percent
Microaneurysms						
Abnormal	17	7.5	30	19.6	47	12.3
<u>Normal</u>	<u>211</u>	92.5	<u>123</u>	80.4	<u>334</u>	87.7
Total	228		153		381	
			x <sup>2</sup> = 12.50		p less than 0.01	
Hemorrhages						
Abnormal	7	3.1	16	10.5	23	6.0
<u>Normal</u>	<u>221</u>	96.9	<u>137</u>	89.5	<u>358</u>	94.0
Total	228		153		381	
			x <sup>2</sup> = 8.81		p less than 0.01	

Abnormal = definite plus uncertain

For statistical purposes, each job was placed into categories of definitely low exposure (DL, less than 3 ppm), moderate exposure (M, 3 ppm through 10 ppm), definitely high exposure (DH, greater than 10 ppm), and a fourth category, other (O), for a number of workers for whom no exposure data were available. Analysis of prevalence by exposure level, as measured by NIOSH industrial hygienists on two occasions in 1979, shows more retinal microaneurysms and hemorrhages in the exposed group than in the control group at  $p = 0.04$  (see Table 4). There is an apparent increase in incidence of microaneurysms as exposure to carbon disulfide increases from low to medium to high. No such trend is apparent for hemorrhages.

TABLE 4. PREVALENCE OF RETINOPATHY BY EXPOSURE LEVEL

	Total		Nonexposed		Low		Medium		High	
	N	%	N	%	N	%	N	%	N	%
<b>Microaneurysms</b>										
Abnormal	47	12.3	17	7.5	8	17.8	12	18.7	10	22.7
Normal	334	87.7	211	92.5	37	82.2	52	81.3	34	77.3
Total	381		228		45		64		44	
$\chi^2 = 13.08; p \text{ less than } 0.01$										
<b>Hemorrhages</b>										
Abnormal	23	6.0	7	3.1	5	11.1	7	10.9	4	9.1
Normal	358	94.0	221	96.9	40	88.9	57	89.1	40	90.9
Total	381		228		45		64		44	
$\chi^2 = 9.01; p = 0.03$										

RELATIONSHIP OF EYE FINDINGS TO CUMULATIVE EXPOSURE

Analysis of the relationship of eye findings to cumulative or long-term exposure is incomplete.

FASTING BLOOD SUGAR (FBS)

Results of the fasting blood sugar survey are shown in Table 5. As seen, there was no statistically significant difference in FBS between groups in this study.

TABLE 5. FASTING BLOOD SUGAR (FBS)  
(CONTROL VERSUS EXPOSURE GROUP)

	<u>Control</u>	<u>Exposed</u>
N	212	145
FBS (mg/dl)	84.27	86.08
Stand. Dev.	11.85	11.56
P Value = 0.15		

DISCUSSION

Fluorescein angiography is by far the best technique for absolute determination of the type of pathology thought to be associated with chronic carbon disulfide exposure (i.e. microaneurysms and/or small point hemorrhages). This information was known to Sugimoto et al. (1976) when they decided to use only color fundus photography for their study and to record the presence or absence of "small red dots" which comprised both microaneurysms and hemorrhages. They were satisfied that their accuracy of detection of relative retinal abnormalities was as good as fluorescein angiography or the more elaborate systems of grading retinopathy used in Sugimoto's 1976 study.

Fluorescein angiography was not seriously considered for this large scale survey of 446 subjects, despite the evidence to suggest that it produces the best sensitivity and specificity. Time constraints and safety concerns in the field ruled against its use. Retinal photography with monochromatic light source was chosen based on information from Delori and Gragoudas (1977) that claimed "excellent demonstration of the retinal vasculature by monochromatic light at 570 nm". The method used to report findings from the retinal slides depends on the method of photography; where Sugimoto's study (1976) made decisions of severity based on "small red dots", this study made decisions of definite or uncertain microaneurysms and hemorrhages based on "small red dots".

The statistical analysis by control versus exposed shows that the carbon disulfide exposed subjects have a statistically significant increase in microaneurysms when compared to control subjects ( $p$  less than 0.01). The same relationship occurs when hemorrhages are considered ( $p$  less than 0.01). These data seem to confirm the experience of the Japanese researchers who found increased incidences of retinal microaneurysms in CS<sub>2</sub>-exposed workers but contrasts with the experience of the Finnish researchers, who found no increase. The actual proportions are about half as great as those reported in Japan but about equal to those found in Yugoslavia.

The Japanese researchers and others consider diabetic vascular changes to be the pathogenic mechanism underlying retinal changes in carbon disulfide-exposed workers. The lack of difference in FBS between exposed and control groups does not rule out a pre-diabetic state in our exposed workers, since the fasting blood sugar is a rather poor test for that disorder. Glucose tolerance testing (GTT) gives better clinical information about pre-diabetic subjects; and the prednisolone GTT used by Goto et al. is considered even more sensitive; but such testing was not feasible due to the large size of the population and time and location constraints in this NIOSH study.

These findings, while preliminary and incomplete, suggest that low level exposure to carbon disulfide is an etiologic factor in development of retinopathy among U. S. white male workers. Just as Sugimoto et al. were left with no explanation for the differences between Japanese and Finnish workers, we can offer no new conjecture explaining the difference in response between American and Finnish workers. Completion of analysis by cumulative exposure may suggest an explanation based on differences in exposure, but the difference shown so far does not appear to be clinically important. Analysis of the relationship between serum lipids, also measured during this study, and presence or absence of retinal changes may help define the pathogenesis of the abnormalities.

The wider biological significance of retinal changes is also not as clear as one would wish. Their presence is certainly not reassuring, particularly when seen in the light of many studies showing an excess of cardiovascular deaths (Hernberg et al., 1970) in carbon disulfide workers and of some studies showing significant renal vascular disease as well, but they are not, of themselves, a disease. If these findings withstand the analyses still pending, they will constitute good support for the proposed revision in U. S. permissible exposure levels downward from 20 ppm. Since the group thought to have exposures less than 3 ppm had a prevalence of both aneurysms and hemorrhages

not significantly different from the medium and definitely high exposure groups our results provide no assurance that the NIOSH-proposed 1 ppm standard will be safe. We are reasonably sure a cohort of workers in the United States who have long-term uncomplicated exposures to carbon disulfide in the range of 1-3 ppm cannot be found, so future research should be directed toward determining the pathophysiology of these changes, explaining the differences in responses between nations, and determining upper limits of safe exposure through animal experimentation.

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EPIDEMIOLOGY OF REFINERY WORKERS WITH  
SOLVENT-RELATED EXPOSURES

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INTRODUCTION

The concern for workers with chronic solvent-related exposures has become a subject of increasing interest (McMichael et al., 1975; Browning, 1965). Because of its ubiquitous nature in petrochemical industries, benzene has received the most attention, especially its possible insults to the hematopoietic system. Numerous case reports and several epidemiologic studies have attempted to establish the leukemogenicity of benzene (Vigiani, 1976; Aksoy, 1974; Infante et al., 1977; Thorpe, 1974; Ott et al., 1978); however, the results have not been consistent.

This study has been undertaken to evaluate the health experience of refinery workers employed in solvent-related processes or operations over a total period of 44 years. The solvents involved are benzene, toluene and methyl ethyl ketone (MEK).

The paper is divided into two parts: the first part describes retrospective cohort studies of benzene workers and lubricating-solvent-dewaxing workers, with the observation period starting at the time operation of these two units began in 1952 and 1935, respectively. The second part is an evaluation of the medical surveillance system for benzene workers which has been instituted since 1959.

RETROSPECTIVE MORTALITY STUDY OF "BENZENE" WORKERS AND "LUBRICATING-SOLVENT-DEWAXING" WORKERS

Two groups of workers from a large refinery have been selected for the study of their potential exposures to different solvents. One group is the chemical workers in benzene and related units and the other is workers in the lubricating-solvent-dewaxing operation. The mortality experience of these two groups will be described and analyzed in terms of standardized mortality ratios (SMRs) with the U. S. general population as the standard.



## DEFINITION OF THE COHORT

The workers included in the benzene cohort worked directly on the benzene, ethylene, aromatic distillate hydrogenation (ADH) or cumene units in this refinery. Benzene is produced in the benzene unit consisting of a platformer which makes benzene and toluene, and a Udex extractor which separates the two chemicals. The operators on the benzene unit also operate the cyclohexane unit to produce cyclohexane from benzene. Benzene is also used as feedstock for the cumene unit. Various depropanizer overhead streams are directed to the ethylene unit from throughout the refinery complex, where they are cracked to produce ethylene as well as ethane and methane. By side-reactions, aromatic compounds are produced which go to the ADH unit to upgrade the benzene and toluene contents by hydrogenation of olefinic impurities, and by separating the resulting products. Some groups of workers also operate the hydrogen producing unit.

The ethylene unit began operation on 09/15/52; benzene, 09/14/59; ADH, 05/03/65; and cumene, 01/05/70.

The workers in the lubricating-solvent-dewaxing cohort started their operation on 06/14/35. The solvent used in the lubricating-solvent-dewaxing process was, initially, a mixture of benzene and methyl ethyl ketone. Benzene was later replaced by toluene in 1945. Among those working in the lubricating department, workers who were exclusively assigned to the centrifuge or wax chilling sections were deleted from the study group because their operations were separate from those of the dewaxing units.

## EXPOSURE DATA

Data of exposure to benzene were only available between 1973 and 1979. The available personnel sampling exposure data has been compiled and shown in Table 1. The sampling strategy for determining personal exposure by industrial hygienists has usually been based on representative sampling by job assignments. Job classifications selected for sampling are usually those where the highest potential for exposure exists during routine operations. Replications of these job classifications were then sampled to characterize the exposure.

TABLE 1. SUMMARY OF BENZENE CONCENTRATION FROM  
EXPOSURE SAMPLES  
(8 Hour Time Weighted Average)

1973 - 1979		
Benzene Concentration	Number of Individual Samples	Percent
Less than 0.1 ppm	161	32
0.1 - 0.99 ppm	220	44
1.0 - 4.99 ppm	93	18
5.0 ppm or more	30	6
TOTAL	504	100.0

From the data available at the time of analysis it should be noted that 76% of those sampled between 1973-1979 were exposed to an 8-hour time weighted average (TWA) of less than 1 ppm benzene and 94% were within 5 ppm benzene.

Earlier workers, prior to 1973, may have had higher exposure. However, the extent to which they had been exposed could only be speculative.

Industrial hygiene measurement of lubricating-solvent-dewaxing workers is limited. In 1977, exposure was recorded for a field sampling of three shifts over a twenty-four hour period. The overall average exposure measured was as follows:

Hexane	0.02 ppm
Benzene	0.05 ppm
Toluene	0.48 ppm
Methyl Ethyl Ketone	0.17 ppm
Methyl Isobutyl Ketone	0.00 ppm

## METHODOLOGY

Copies of all personnel records since the start of the refinery operation in 1901 were made and pertinent demographic and work history information were screened according to the cohort definition described.

Of approximately 20,000 employee records collected and screened, 443 have been included in the benzene worker cohort and 1,005 included in the lubricating-solvent-dewaxing cohort.

Standard epidemiologic procedures of ascertaining the vital status of the two cohorts were followed. They included matching the cohort employees with rosters of current employees and annuitants, tracing by refinery personnel, obtaining information from the Social Security Administration and from the Texas Motor Vehicle Bureau, and collecting death certificates from the vital statistics departments of individual states. The underlying cause of death was coded to the 8th revision of the ICD by a trained nosologist according to the rules in effect at the time of death.

All of the above data were stored in the computer and statistical analysis was largely based on a computer program developed by Monson (1974).

## RESULTS

### BENZENE COHORT

Of the benzene cohort (N = 443 employees with a total of 9142 person-years), 413 employees were found to be alive, 28 deceased, and 2 unknown as of 1976. Follow-up was 99.5% successful. Forty percent of the cohort were hired before 1950 (Table 2) and one-third of the cohort had been terminated before the end of the study. Fifty-five percent of the cohort worked more than 20 years (Table 3).

TABLE 2. DISTRIBUTION BY YEAR OF HIRE OF THE BENZENE  
AND THE LUBRICATING-SOLVENT-DEWAXING COHORT

Year of Hire	Benzene Cohort		Lubricating-Solvent-Dewaxing Cohort	
	Number	Percent	Number	Percent
Before 1929	32	7.2	208	20.7
1930-1939	23	5.2	78	7.8
1940-1949	123	27.8	338	33.6
1950-1959	104	23.5	283	28.2
1960-1969	70	15.8	30	3.0
1970-1976	91	20.5	68	6.8
TOTAL	443	100.0	1005	100.1

TABLE 3. DISTRIBUTION BY LENGTH OF EMPLOYMENT OF THE  
BENZENE AND THE LUBRICATING-SOLVENT-DEWAXING COHORT

Length of Employment	Benzene Cohort		Lubricating-Solvent-Dewaxing Cohort	
	Number	Percent	Number	Percent
6 Months	5	1.1	8	0.8
6 - 11 Months	20	4.5	12	1.2
1 - 4 Years	53	12.0	67	6.7
5 - 9 Years	90	20.3	97	9.6
10 - 14 Years	14	3.2	34	3.4
15 - 19 Years	16	3.6	50	5.0
20 - 24 Years	80	18.1	185	18.4
25 - 29 Years	70	15.8	176	17.5
30 - 34 Years	44	9.9	173	17.2
35 - 39 Years	26	5.9	108	10.8
40 - 44 Years	21	4.7	74	7.4
45 - 49 Years	4	0.9	21	2.1
TOTAL	443	100.0	1005	100.1

Tables 4, 5, and 6 show the observed and expected deaths by cause, SMRs, and their 95% confidence limits for white males, black males, and all males in the benzene cohort, respectively. Death certificates have not been obtained for two of the 28 total deaths. These two deaths were included in the overall mortality analysis but not in the cause-specific SMR computation. A total of 20 deaths were observed among the white male benzene workers whereas 46.88 were expected based on the total U. S. white male mortality experience (Table 4). The resultant SMR of 43 is lower than expected with

TABLE 4. OBSERVED AND EXPECTED DEATHS BY CAUSE,  
SMRs, AND THEIR 95% CONFIDENCE LIMITS FOR  
WHITE MALES IN BENZENE COHORT  
N = 354

Cause of Death	Observed Deaths	Expected Deaths	SMR	Lower Limit	Upper Limit
All causes	20	46.88	43**	26	66
All cancers (140-209)	5	8.47	59	19	138
Cancer of Digestive System (150-159)	1	2.27	44	1	245
Cancer of Large Intestine (153)	1	0.70	144	2	799
Cancer of Respiratory System (160-163)	2	2.82	71	8	256
Cancer of Lung (162-163)	2	2.66	75	8	272
Cancer of Brain (191)	1	0.36	277	4	1542
Diseases of Circulatory System (390-458)	10	21.03	48*	23	87
Arteriosclerotic Heart Disease (410-413)	6	15.17	40*	14	86
Cerebrovascular Disease (430-438)	2	2.33	86	10	311
Nonmalignant Respiratory Diseases (460-519)	1	2.34	43	1	238
Pneumonia (480-486)	1	0.93	108	1	600
Diseases of Digestive System (520-577)	1	2.71	37	0	205
Accidents, Poisonings & Violence (E800-E999)	3	7.85	38	8	112
Accidents (800-949)	2	5.56	36	4	130
Suicide (950-959)	1	1.66	60	1	334

\* Statistically significant at 0.05 level

\*\* Statistically significant at 0.01 level

TABLE 5. OBSERVED AND EXPECTED DEATHS BY CAUSE,  
SMRs, AND THEIR 95% CONFIDENCE LIMITS FOR  
BLACK MALES IN BENZENE COHORT  
N = 89

Cause of Death	Observed Deaths	Expected Deaths	SMR	Lower Limit	Upper Limit
All causes	8	21.11	38**	16	75
All cancers (140-209)	2	3.04	66	7	237
Cancer of Digestive System (150-159)	1	0.99	101	1	565
Cancer of Stomach (151)	1	0.26	382	5	2127
Cancer of Respiratory System (160-163)	1	1.01	99	1	551
Cancer of Larynx (161)	1	0.06	1667	22	9275
Disease of Circulatory System (390-458)	4	8.01	50	13	128
Arteriosclerotic Heart Disease (410-413)	2	3.83	52	6	189

\*\* Statistically significant at 0.05 level

TABLE 6. OBSERVED AND EXPECTED DEATHS BY CAUSE,  
SMRs, AND THEIR 95% CONFIDENCE LIMITS FOR  
ALL MALES IN BENZENE COHORT  
N = 443

Cause of Death	Observed Deaths	Expected Deaths	SMR	Lower Limit	Upper Limit
All causes	28	67.99	41**	27	60
All cancers (140-209)	7	11.51	61	24	125
Cancer of Digestive System (150-159)	2	3.26	61	7	222
Cancer of Stomach (151)	1	0.70	143	2	795
Cancer of the Large Intestine (153)	1	0.87	115	2	640
Cancer of Respiratory System (160-163)	3	3.83	78	16	229
Cancer of Larynx (161)	1	0.19	526	7	2928
Cancer of Lung (162-163)	2	3.60	56	6	201
Cancer of Brain (191)	1	0.41	244	3	1357
Diseases of Circulatory System (390-458)	14	29.04	48**	26	81
Arteriosclerotic Heart Disease (410-413)	8	19.00	42**	18	83
Cerebrovascular Disease (430-438)	2	4.02	50	6	180
Nonmalignant Respiratory Diseases (460-519)	1	3.57	28	0	156
Pneumonia (480-486)	1	1.66	60	1	335
Diseases of Digestive System (520-577)	1	3.90	26	0	143
Accidents, Poisonings & Violence (E800-E999)	3	11.88	25**	5	74
Accidents (800-949)	2	7.74	26*	3	93
Suicide	1	1.87	53	1	298

\* Statistically significant at 0.05 level

\*\* Statistically significant at 0.01 level

statistical significance at the 0.01 level. This deficit of 57% came primarily from diseases of the circulatory system (SMR = 48,  $p < 0.05$ ) or, more specifically, arteriosclerotic heart disease (SMR = 40,  $p < 0.05$ ). Five cancer deaths occurred in this group, while 8.47 were expected. However, this deficit is not significant. In fact, none of the site-specific cancer SMRs computed is significantly different from unity. It should also be noted that none of the observed deaths was due to leukemia or lymphopoietic cancer.

Among the 89 black males in the benzene cohort, eight deaths were observed compared to an expectation of 21.11 (Table 5). The overall SMR is 38, significantly decreased at the 0.05 level. None of the cause specific SMRs are significant.

To partially overcome the problem of small sample size, the whites and blacks were combined in Table 6. A total of 28 deaths were observed in the benzene cohort, compared to the expected deaths of 67.99. The overall SMR is 41, significantly decreased at the 0.01 level. The deficits in diseases of the circulatory system (SMR = 48,  $p < 0.01$ ) or, more specifically, arteriosclerotic heart disease (SMR = 42,  $p < 0.01$ ) were responsible for most of the deficit in the overall mortality. Seven cancer deaths were observed while 11.51 were expected. In particular, no leukemia deaths were identified in the benzene cohort.

#### LUBRICATING-SOLVENT-DEWAXING COHORT

This cohort (N = 1005 with a total person-year observation of 27,015) includes 25 employees with an unknown status (follow-up rate: 97.5%). Sixty-two percent of the cohort was hired before 1950 (Table 2) and 52% of the cohort had been terminated before the end of the study. Seventy-three percent of the cohort had worked more than 20 years at the plant (Table 3).

Tables 7, 8, and 9 show the observed and expected deaths by cause, SMRs, and their 95% confidence limits for white males, black males, and all males in the lubricating-solvent-dewaxing cohort, respectively.

Table 7 shows that the overall mortality for white male employees is significantly less than the expected with 107 observed and 149.42 expected (SMR = 72,  $p < 0.01$ ). Based on the U. S. white male mortality rates, none of the cause-specific SMRs is outside the expected range. Twenty-three cancer deaths were observed while 27.16 would have been expected. In particular, only one death each was due to leukemia and cancer of the prostate - slightly, but not significantly, less than the expected deaths.

Among the blacks in the lubricating-dewaxing cohort, in addition to total mortality, significant deficits were also found in cancer of the digestive system, (SMR = 59,  $p < 0.05$ ), diseases of the circulatory system (SMR = 59,  $p < 0.01$ ), cerebrovascular diseases (SMR = 47,  $p < 0.05$ ) and accidents (SMR = 19,  $p < 0.01$ ) (Table 8). Two deaths from cancer of the bone were observed where only 0.13 was expected. The corresponding SMR of 1494 is statistically significant at the 0.05 level. Six deaths were observed from cancer of the prostate, whereas 2.80 were expected. However, the corresponding SMR of 214 did not reach statistical significance.

When both races were combined in Table 9, the mortality pattern remains basically the same as that among the blacks. A total of 190 deaths were observed with 311.51 expected and 40 deaths due to cancer were observed with 51.41 expected. In addition, deficits in nonmalignant respiratory diseases and digestive system diseases were also significant at the 0.05 level. A total of three deaths were due to cancer of the bone, and with only 0.31 expected, resulting in a SMR of 968 ( $p < 0.01$ ). Seven deaths from prostatic cancer were identified, compared to 4.11 expected. However, the increased SMR for prostatic cancer (SMR = 170) is not statistically significant. Two leukemia deaths were observed while 1.81 were expected with an SMR of 110.

TABLE 7. OBSERVED AND EXPECTED DEATHS BY CAUSE, SMRs,  
AND THEIR 95% CONFIDENCE LIMITS FOR WHITE MALES IN  
LUBRICATING - DEWAXING COHORT  
N = 700

Cause of Death	Observed Deaths	Expected Deaths	SMR	Lower Limit	Upper Limit
All causes	107	149.42	72**	59	87
All cancers (140-209)	23	27.16	85	54	127
Cancer of Digestive System (150-159)	7	7.82	90	36	184
Cancer of Large Intestine (153)	1	2.34	43	1	238
Cancer of Rectum (154)	1	0.94	106	1	591
Cancer of Pancreas (157)	3	1.48	203	41	592
Cancer of Respiratory System (160-163)	5	8.79	57	18	133
Cancer of Lung (162-163)	5	8.26	61	20	141
Cancer of Bone (170)	1	0.18	555	7	3088
Cancer of Skin (172)	1	0.54	185	2	1029
Cancer of Prostate (185)	1	1.31	76	1	426
Cancer of Kidney (189)	1	0.71	142	2	789
Lymphatic & Hematopoietic Cancer (200-209)	5	2.94	170	55	397
Hodgkin's Disease (201)	1	0.51	196	3	1088
Leukemia & Aleukemia (204-207)	1	1.19	84	1	468
Diseases of Circulatory System (390-458)	52	71.47	73*	54	95
Arteriosclerotic Heart Disease (410-413)	41	50.01	82	59	111
Cerebrovascular Disease (430-438)	6	8.67	69	25	151
Nonmalignant Respiratory Diseases (460-519)	5	7.97	63	20	146
Pneumonia (480-486)	3	3.20	94	19	274
Emphysema (492)	1	1.97	51	1	283
Diseases of Digestive System (520-577)	4	8.26	48	13	124
Cirrhosis of Liver (571)	1	4.14	24	0	134
Accidents, Poisonings & Violence (E800-E999)	14	20.15	69	38	117
Accidents (800-949)	7	14.31	49	20	101
Motor Vehicle Accidents (810-827)	3	6.70	45	9	131
Suicide (950-959)	5	4.40	114	37	265

\* Statistically significant at 0.05 level.

\*\* Statistically significant at 0.01 level.

TABLE 8. OBSERVED AND EXPECTED DEATHS BY CAUSE, SMRs,  
AND THEIR 95% CONFIDENCE LIMITS FOR BLACK MALES IN  
LUBRICATING-DEWAXING COHORT  
N = 305

Cause of Death	Observed Deaths	Expected Deaths	SMR	Lower Limit	Upper Limit
All causes	83	169.09	51**	41	63
All cancers (140-209)	17	24.25	70	41	112
Cancer of Digestive System (150-159)	2	8.68	23*	3	83
Cancer of Stomach (151)	1	2.70	37	0	206
Cancer of Pancreas (157)	1	1.35	74	1	411
Cancer of Respiratory System (160-163)	4	6.69	60	16	153
Cancer of Lung (162-153)	4	6.18	65	17	166
Cancer of Bone (170)	2	0.13	1494*	168	5329
Cancer of Prostate (185)	6	2.80	214	78	466
Cancer of Brain (191)	1	0.28	352	5	1956
Lymphatic & Hematopoietic Cancer (200-209)	1	1.65	61	1	338
Leukemia & Aleukemia (204-207)	1	0.62	160	2	893
Diabetes Mellitus (250)	4	2.29	174	47	446
Diseases of Circulatory System (390-458)	43	73.43	59**	42	79
Arteriosclerotic Heart Diseases (410-413)	30	32.18	93	63	133
Cerebrovascular Disease (430-438)	8	17.12	47*	20	92
Nonmalignant Respiratory Diseases (460-519)	4	10.06	40	11	102
Pneumonia (480-486)	2	6.22	32	4	116
Diseases of Digestive System (520-577)	3	7.03	43	9	125
Cirrhosis of Liver (571)	2	2.68	75	8	269
Disease of Genito-Urinary System (580-629)	4	6.40	63	17	160
Accidents, Poisonings & Violence (E800-E999)	4	17.82	22**	6	57
Accidents (800-949)	2	10.75	19**	2	67
Motor Vehicle Accidents (810-827)	1	4.13	24	0	135
Suicide (950-959)	1	0.89	113	1	627

\* Statistically significant at 0.05 level.

\*\* Statistically significant at 0.01 level.



TABLE 9. OBSERVED AND EXPECTED DEATHS BY CAUSE,  
SMRs, AND THEIR 95% CONFIDENCE LIMITS FOR ALL MALES IN  
LUBRICATING-DEWAXING COHORT  
N = 1005

Cause of Death	Observed Deaths	Expected Deaths	SMR	Lower Limit	Upper Limit
All causes	190	311.51	61**	53	70
All cancers (140-209)	40	51.41	78	56	106
Cancer of Digestive System (150-159)	9	16.50	55	25	104
Cancer of Stomach (151)	1	4.34	23	0	128
Cancer of Large Intestine (153)	1	3.91	26	0	142
Cancer of Rectum (154)	1	1.59	63	1	350
Cancer of Pancreas (157)	4	2.83	141	38	362
Cancer of Respiratory System (160-163)	9	15.48	58	27	110
Cancer of Lung (162-163)	9	14.44	62	28	118
Cancer of Bone (170)	3	0.31	968**	195	2828
Cancer of Skin (172)	1	0.68	147	2	818
Cancer of Prostate (185)	7	4.11	170	68	351
Cancer of Kidney (189)	1	1.08	93	1	515
Cancer of Brain (191)	1	1.29	78	1	431
Lymphatic & Hematopoietic Cancer (200-209)	6	4.59	131	48	285
Hodgkin's Disease (201)	1	0.71	141	2	784
Leukemia & Aleukemia (204-207)	2	1.81	110	12	399
Diabetes Mellitus (250)	4	4.36	92	25	235
Diseases of Circulatory System (390-458)	95	144.90	66**	53	80
Arteriosclerotic Heart Disease (410-413)	71	82.19	86	67	109
Cerebrovascular Disease (430-438)	14	25.79	54*	30	91
Nonmalignant Respiratory Diseases (460-519)	9	18.03	50*	23	95
Pneumonia (480-486)	5	9.42	53	17	124
Emphysema (492)	1	2.84	35	0	195
Diseases of Digestive System (520-577)	7	15.29	46*	18	94
Cirrhosis of Liver (571)	3	6.82	44	9	129
Disease of Genito-Urinary System (580-629)	4	8.90	45	12	115
Accidents, Poisonings & Violence (E800-E999)	18	37.97	47**	28	75
Accidents (800-949)	9	25.06	36**	16	68
Motor Vehicle Accidents (810-827)	4	10.83	37*	10	95
Suicide (950-959)	6	5.29	113	41	247

\* Statistically significant at 0.05 level

\*\* Statistically significant at 0.01 level

No changes in results were observed when the white male mortality experience was analyzed by length of employment, except that the significant deficit in all causes disappeared in the group of white lubricating-dewaxing workers with at least 25 years of employment. Among the blacks, the SMR for cancer of the bone increased with minimum length of employment: 1594 ( $p < 0.05$ ), 1861 ( $p < 0.05$ ), and 2713 ( $p < 0.01$ ) for those with at least 5, 15, and 25 years of employment, respectively. However, a similar trend is not observed in the combined analysis by length of employment for both white and black males in the lubricating-dewaxing cohort.

## DISCUSSION

### BENZENE COHORT

Overall mortality analysis for the benzene cohort in this study revealed no significant excess. On the contrary, mortality from all causes, from diseases of the circulatory system, from arteriosclerotic heart disease, and from accidents were significantly lower than the expected number based on a comparable group of U. S. males. In particular, no observed death was due to leukemia, a condition of greatest interest in this benzene study. Based on the 9142 person-years of observation, there is a 50% probability of detecting a 19-fold risk of leukemia and a 30% probability of detecting a 5-fold risk, both at the  $p = 0.05$  level.

As mentioned above, no leukemia was observed in the benzene cohort. Even if we assume there was one leukemia observed, the relative risk would be equal to 0.73 and the 95% confidence interval would be 0.76 to 7.039.

Despite the small sample size of the benzene cohort, a relative risk of greater than 7 is highly unlikely. From the present study the relative risk of developing leukemia is nearly zero.

Using an internal control for comparison (i.e., workers from the same plant employed during the same period but not in benzene related operations), again no adverse mortality was found in the benzene workers (the results will be presented in a separate paper).

As described in the definition, the benzene cohort in this study does not include maintenance workers who might have been exposed to benzene on an occasional basis. The experience of the maintenance workers and other workers in the plant will be presented in a separate paper.

### LUBRICATING-SOLVENT-DEWAXING COHORT

Lubricant-dewaxing workers have only been studied by Enterline (1978). The process used in this study is similar to the one in this study and has involved solvent dewaxing with a mixture of methyl ethyl ketone, benzene (discontinued in 1952), and toluene. Among the 305 workers studied between 1948 and 1975, there were 27 observed deaths with 2 deaths due to cancer of the prostate. The expected death from prostate cancer was 0.21, so the SMR was 952, statistically significant at the 0.05 level. However, the number involved is stated to be too small to make any definite conclusions.

Hendricks (Hendricks et al., 1959) reported that wax pressmen with 10 years or more of service showed a significant increase in scrotal cancer. The work of wax pressmen involved passing the chilled wax containing distillate through plate and frame presses. Wax was then manually removed from the plates and further purified by "sweating" acid treating neutralizing and filtration.

A more recent study reported the finding of no excess cancer mortality except for leukemia and kidney cancer in the newspaper web pressmen (Paganini-Hill et al., 1980). Seven leukemia deaths were observed and only 2.8 deaths were expected, resulting in an SMR of 247 which is statistically significant at the 5% level. Five deaths from cancer of the kidney were observed among the pressmen, whereas only 1.6 deaths were expected (SMR = 303, significant at the 5% level). Benzene was used in the pressroom as a solvent and in ink formulation and still may have been present as a process impurity. Toluene and other solvents have subsequently been substituted for benzene.

Upon analysis of the overall mortality of the present study, the lubricating-solvent-dewaxing cohort demonstrated a favorable outcome. In addition, mortality from diseases of the circulatory system, from cerebrovascular disease, from non-malignant respiratory diseases, from diseases of the digestive system, and from accidents were all significantly less than expected.

Seven deaths were due to cancer of the prostate with 4.11 expected. The excesses were primarily seen in the black. The resulting SMR of 170, though increased, is not statistically significant. Analysis by race and minimum length of employment did not show any significant excess in prostatic cancer mortality, although the SMR for prostatic cancer increases slightly with the minimum length of employment (5 years or more, 15 years or more, and 25 years or more). The SMR for prostatic cancer of black males employed 25 years or more is 260 with a 95% confidence interval of 95 to 565. In view of the similar results observed by Enterline, it is prudent to pursue this lead and verify its validity.

Three cases of bone cancer were observed while 0.31 were expected. (SMR = 968,  $p < 0.05$ ). The three cases were listed on the death certificate as: Case 1) chondrosarcoma of right femur; Case 2) generalized carcinomatosis due to cancer of the spine, and; Case 3) carcinoma of the spine with metastasis.

Since bone tissues are derived from mesenchymal tissues, primary cancers of the bone are called sarcomas. In contrast, tissues of the internal organs are derived from epithelial tissues and cancers of these epithelial tissues are called carcinomas. Thus, the diagnosis of carcinoma of the spine is either a misnomer or a description of a metastatic cancer to the spine. Indeed, Case 2 had a primary lung cancer which metastasized to the spine. Case 3 had metastatic carcinoma to the spinal canal with bone destruction and obstruction at the T11 vertebra. The cells of the tumor appeared to be not of bone, lung, or prostate but of glial origin.

Thus, except for Case 1, all of the bone cancer as coded under the 8th ICD revision should not have been bone cancer. This nomenclature problem was recognized and corrected in the 9th ICD revision that took effect in 1979. Under this new revision, the last two cases above would be coded as metastatic cancer (to the spine) from an unspecified site.

In summary, examination of mortality experience of benzene workers (1952-1976) and solvent-dewaxing workers (1935-1976) showed a favorable overall mortality (SMR = 41 and 61, respectively). None of the benzene workers developed leukemia. The solvent-dewaxing workers showed some excess of prostatic cancer, particularly in black males, but the observed number was small and the excess was not statistically significant.

### MEDICAL SURVEILLANCE PROGRAM OF BENZENE WORKERS

While reduction of the workers' exposure to a minimum has been continuously sought, the health of benzene workers requires close monitoring in order to detect any adverse effects as a result of benzene exposure. Even though such a principle is widely accepted, the protocol to administer cost effect "screening procedures" is far from settled. Of particular controversy is the utility of the screening of blood for any sign of blood dyscrasia. Townsend et al. (1978) studied the health examination findings of 282 men occupationally exposed to benzene and found slight decreases in mean total bilirubin and RBC values when compared to a control group, but these changes were not regarded as clinically significant. Fishbeck et al. (1978) found no persistent hematologic effects except for a slight reduction in hemoglobin and an increase in the mean corpuscular volume (MCV) of ten employees with chronic benzene exposure exceeding 25 ppm, eight-hour TWA for several years. However, Greenburg et al. (1939) and Aksoy et al. (1971) found significant abnormalities in hematologic results of workmen with reportedly high level exposure to benzene (up to 210 ppm).

This paper presents data from a subset of the same refinery workers already presented. Blood screening has been conducted in this refinery for a period of 21 years.

### SUBJECTS AND METHODS OF STUDY

As part of the medical surveillance program of morbidity and mortality at the refinery, blood from every benzene worker was tested four times a year starting in 1959, for hemoglobin, hematocrit, white blood cell count with differential, platelet count, bleeding and clotting time. Frequency of examination was later reduced to once a year in the early 1960's. With the advent of the Autoanalyzer and Coulter counter, a SMA12 or SMAC and a CBC have been added.

A benzene worker was defined annually by the supervisor of the units and included all workers ever assigned to one of the following units: Benzene, ADH, Hydrogenation, Ethylene, Polymerization, Cumene, Pump Houses, Loading Shop and Docks, and Barges of the Bulk Oil Department.

It should be noted that both operators and maintenance workers have been included by this definition.

## OUTCOME OF WORKERS IN THE MEDICAL SURVEILLANCE PROGRAM

Results of the hematology and blood chemistry profile are shown in Table 10 in terms of mean, standard deviation, values at 5th and 95th percentiles, and the number of examinations recorded and analyzed. As a group, the workers showed normal values and no tendency of pancytopenia (or aplastic anemia) or bleeding or clotting deficit, judged from the mean and 5th and 95th percentile values in the hematology data profile. Results of the blood chemistry profile were also within normal limits including bilirubin, which should have increased if hemolytic anemia occurred or liver function was impaired (Table 11). A small portion of the values falling within the lower or upper 5% might have been considered clinically "abnormal", but most results obtained on repeated testing were not replicated and, therefore, clinically judged to be normal without further follow-up. Most of these discrepancies resulted from variations in laboratory techniques and biological systems, although some of the abnormal results could have been real but were transient and reversible. In one case, repeated blood tests led to an early diagnosis of multiple myeloma.

TABLE 10. SUMMARY OF HEMATOLOGY DATA PROFILE

	Mean	S.D.	Values at 5%	Values at 95th%	Number of Examinations
WBC/cu mm	6,952	1,891	4,300	10,300	1,404
polymorphonuclear	4,177	1,456	2,112	6,588	1,396
lymphocyte	2,406	782	1,350	3,860	1,396
Hemoglobin (in gm % blood)	14.8	1.1	13.0	16.7	1,404
Hematocrit (%)	44.4	3.7	39.0	50.4	1,399
RBC ( $\times 10^6$ /cu mm)	5.0	0.4	4.4	5.6	602
Platelet ( $\times 10^3$ /cu mm)	293.0	97.0	160.0	459.0	1,085
Bleeding time (minute)	1.64	0.80	0.62	3.0	1,001
Clotting time (minute)	6.00	1.00	3.38	6.58	1,002

Data have been collected between 1959-1979. The number of examinations on individuals varies.

TABLE 11. SUMMARY OF BLOOD CHEMISTRY PROFILE

	Mean	S.D.	5th%	95th%	Number of Examinations
Glucose (mg %)	94.1	17.4	73	118	903
BUN (mg %)	15.1	4.2	10	21	902
Uric Acid (mg %)	6.3	1.3	4.5	8.4	902
Calcium (mg %)	9.9	0.5	9.1	10.5	895
Phosphorus (mg %)	3.3	0.5	2.4	4.1	895
Cholesterol (mg %)	207	41	144	275	897
Triglyceride (mg %)	134	84	48	287	266
SGOT (units)	30.6	15.2	14	54	902
SGPT (units)	31.1	23.1	9	56	272
Bilirubin Total (mg %)	0.65	0.27	0.2	1.1	902
Bilirubin Direct (mg %)	0.15	0.13	0.1	0.2	245
Bilirubin Indirect (mg %)	0.56	0.32	0.1	1.0	266
Total Protein (gm %)	7.2	0.5	6.4	7.9	904
Globulin (gm %)	2.7	0.5	2.0	3.2	270
Albumin (gm %)	4.4	0.4	3.8	4.9	903

Data have been collected between 1970-1979. Number of examinations vary on individuals.

Of 303 employees who have received medical surveillance, a total of 11 deaths have occurred as of the end of 1979 while 21.46 deaths are expected (SMR = 51). Causes for these 11 deaths include 7 from coronary heart diseases (9.68 expected), 1 from cirrhosis of the liver (0.90 expected), 1 from natural cause, and 2 from cancer (4.42 expected). The 2 cancer cases are malignant melanoma and multiple myeloma. As of 1979, none of the reported deaths has been ascribed to leukemia nor have any living employees developed leukemia, lymphoma or other blood dyscrasia--the focal point of chronic benzene exposure (Vianna and Polan, 1979).

#### DISCUSSION

The benefits of the medical surveillance system are many. They include: creation of baseline data for future comparisons; provision for trend analysis for group or subgroups; early detection of adverse health effects; medical benefits to the employee; and increased awareness for health maintenance and promotion.

From the clinical standpoint, early detection of adverse health effects is probably the most important justification for a medical surveillance program. However, the experience of the program described in this paper raises questions concerning the effectiveness of screening for such a purpose.

As in every biological phenomenon, the screening results varied from individual to individual and among same individuals from year to year. These variations have been intensely confounded by different laboratory standards

and practices throughout the years. Some of them are related to the advancement of technology, such as the advent of the Coulter counter. In evaluating these results, the absence of a comparable "healthy population" standard further hampers an objective analysis. A study by Frederik (1974) of approximately 20,000 employees indicated that the usual hematologic normal ranges based on hospital patients did not accurately represent an employed population. Thus, even though in several instances the data may be judged to be marginal or even "abnormal" by hospital standards, they could well be within the variations caused by factors other than biological.

It should be realized that the predictive value (Vecchio, 1968) of each test or a battery of tests is not well established for test results when they are marginal. Only when values are overtly abnormal can one conclude that health status may be compromised and in serious jeopardy. (For example: values of a white blood count of 4,000 or 11,000 could be clinically judged to be marginal but well within expected variation. On the other hand, this is not the case when WBC exceeds 50,000 with the appearance of premature cells.)

In a proposed OSHA standard for benzene (1978) which was struck down by the Supreme Court, the employer should have had the test results of the employee evaluated by a hematologist if:

- 1) RBC, Hgb or platelet count varied more than 15% above or below the employees' most recent values;
- 2) RBC was below 4.4 million or above 6.3 million per  $\text{mm}^3$  (for males);
- 3) Hgb was below 14 gm % or above 18 gm % (males);
- 4) WBC was below 4,200 or above 10,000;
- 5) Platelet count was below  $140 \times 10^3$  or above  $440 \times 10^3$ .

It should be noted that the OSHA recommended evaluation by a hematologist was not based on any probability projection. Furthermore, there was no estimate as to the proportion of tests that would be referred. Using the data presented in this paper it was calculated that 45% of the test results would have caused referrals to hematologists, had the OSHA standard been in effect since 1959. The economic cost, the mental stress, and the drain on the specialists' time and resources resulting from such "untested" procedures are enormous.

However, the potential value of medical surveillance in the area of early case findings still cannot be overemphasized, if physicians treat the examination results with vigilance and prudent judgement.

In the present example, as the result of hematologic screening, one patient was suspected of and later proved to be suffering from multiple myeloma. Other less serious conditions have also been diagnosed. However, not all early interventions proved to be totally effective, as in the case of the patient with multiple myeloma.

There has been considerable debate as to the wisdom of annual physicals. It is obvious that the yield may not be high when the screened population is young, healthy, and asymptomatic. However, an employed population of a petrochemical industry with potential exposure risk probably needs special attention, even though the majority of them may seem to be healthy.

In the evaluation of an occupational medical surveillance program, a different set of criteria may be necessary to evaluate the conventional cost-effectiveness or risk/benefit ratio. The surveillance program in occupational medicine attempts to satisfy more than the simple cost-effectiveness criteria. It tries to assure the realization of the ideal goal of a "risk-free" environment with no ill health resulting from occupational hazards, and data created in the surveillance program for baseline or follow-up could serve multiple purposes including clinical, epidemiologic and legal uses.

However, the occupational medical surveillance program should be continuously evaluated and the predictive value of each test or battery of tests periodically sought to improve its efficiency and to define priorities. Eventually all of the findings should be translated into actions, whether such actions are health education for a change of lifestyle, process engineering changes to reduce exposure, or clinical referral and/or intervention to prevent illness and disability.

In summary, a medical surveillance program of benzene workers has been described, with special emphasis on the effectiveness of laboratory tests. Evaluation of 21-year data shows no significant changes in blood indices of these workers as the result of chronic exposure to benzene.

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#### ACKNOWLEDGEMENT

The authors want to thank Dave Bennett and Jim Goemmer for computer support, Carol Bennett and Yolanda Sanchez for ICD coding, Nancy Weiss and Nora Schwartzkopf for manuscript preparation.

## OPEN FORUM

MR. VERNOT (University of California, Irvine): My question is to Dr. Leffingwell. I was a little uncertain about the change in methodology that occurred. If I understood you correctly, when you first investigated the number of aneurysms in your control group, you found what you considered to be an inordinately high percentage of about 25%. Did you take a look at the exposed population using that technique, and did you change your criteria for the definition of aneurysms in the final analysis of the data?

DR. LEFFINGWELL (National Institute for Occupational Safety and Health): We did use the same slide reading technique initially on both the exposed and the unexposed groups. The proportions of abnormal slides were similar in both. It was around 25% in both the exposed and the unexposed. The initial reading simply read them as normal, having microaneurysms, or having hemorrhages. In this system you couldn't have both normal and one of the other findings but you could have both hemorrhages and microaneurysms. The revised criteria permitted a definite hemorrhage or an uncertain hemorrhage and a definite microaneurysm or an uncertain microaneurysm to take into account the dots and spots that were not so obviously normal or abnormal. The second reading was by a different ophthalmologist than the one who had done the first or the two who had done the first reading. I think he tended to read all of them a bit more conservatively than the first group did. But certainly there were a great many in the uncertain group after the second evaluation.

MR. VERNOT: Does this imply that the differences occurring between the various national groups may be associated with differences in the methodology for defining aneurysms?

DR. LEFFINGWELL: In the case of the joint Finnish-Japanese study, they took pictures and Dr. Raitta read the Japanese as well as the Finnish cases and sent them on to Dr. Hotta who also read both sets of slides. I don't recall if they actually published a concordance table, but they apparently did agree quite well on overall percentages and that there were essentially no aneurysms in Finland and a lot of them in Japan.

LCDR PITTS (Naval Medical Research Institute): I'm addressing these comments to Dr. Wen. I have had some experience with the SMA12 and the SMAC and with these types of test batteries. One of the problems that occurs is that approximately 40% of the people tested will show one or more abnormality to start with. Another problem with panel screening is that the selection of tests quite often has been an historical selection of tests by the instrument manufacturer and it may have been necessary to accept the battery of tests that were not necessarily a selection of those that you might want to follow. And, finally, there is the problem that many clinical chemistry parameters have a non-normal distribution and there are sex and race differences.

DR. WEN (Gulf Science and Technology Company): I think the point is well made. We are facing the same dilemma. A lot of data have been generated through screening procedures and a high percentage of them are abnormal. From a clinical standpoint, we are obligated to investigate each individual case and to accept or

dismiss abnormal values on the basis of clinical examination. That is almost impossible and therefore, I think the criteria have to be different in a large scale screening program and would have to take different statistical consideration of what is to be considered normal.

LCDR PITTS: Have you all given any thought to the selection of more appropriate tests instead of using the standard 12 tests that were available on the machine that was available to you? Could you say we only want to use 7 of these tests instead of the 12?

DR. WEN: Since they're all available, you can ignore some of the 24 tests if you only want to look at a few. My plea would be to create a data base for a healthy population that we can use for comparisons even though we know a certain percentage are so-called clinically abnormal. In a population they would still be considered normal biological statistical variations and so far there are no data available for us to compare.

DR. HODGE (University of California, San Francisco): Dr. Kaplan, in your big slide of mortality ratios there were data on cancers of the large intestine not including the rectum. On that slide, cancer of the urinary organs had a ratio of approximately 2. Did you comment on that?

DR. KAPLAN (SRI International): I didn't comment on cancer of the urinary organs in my talk because of time considerations but we certainly commented on it extensively in our report and that was one point that we had a lot of concern about. That was a mixture of cancers of the bladder and cancers of the kidney but from what little we know of the etiology of these diseases in humans, factors that are associated with one seem to be associated with the other. For example, cigarette smoking. We felt that this was one of the causes that should be most actively investigated in a future study. We still have only five deaths in a mixed group but I would say that was highly suspicious. We could not do the kind of analysis for consistency that we could with the cancer of the large intestine because of the smaller numbers. For instance, with four union locals and five deaths, it's difficult to say whether it was consistent in all four union locals or if a chance phenomenon was going on. We certainly are concerned about these cancers. Incidentally, the report is available from the project officer and will be published as an NTIS report later.

DR. WEN (Gulf Science and Technology Company): Dr. Kaplan, you made an assumption that the race distribution of the dry cleaner population was the same as the race distribution of those deceased. How good is that assumption and if that assumption was violated in the true race distribution would the results have been somewhat different?

DR. KAPLAN (SRI International): If we had a way to check the assumption we never would have made it. We were stuck with the data we had which is one of the limitations of an epidemiologic study. We also looked at the SMR's under the assumption that everybody was white to compare them with white rates and also under the assumption that everybody was black. We know that neither of these is correct and that the true value will almost always be in between the two. For the causes of death that I presented in the slide, which are the ones of greatest interest, that assumption made very little difference. The estimates

of the SMR's were not very different and the interpretations were not affected. For other causes of death, it really made a big difference. As I recall, for diseases of the genital urinary system, if you used rates based on the assumption that everybody was black, you had a deficit of observed to expected deaths, and if you made the assumption that everybody was white, you had about a two fold excess. Comparing the data, using the assumption we made on racial distribution which was our best estimate, we found the number of observed deaths very close to the number of expected deaths.

DR. COURI (Ohio State University School of Medicine): We have heard many generalizations about the relationships of cancer and environmental or occupational exposure to chemicals. I'd like the panel to comment on this subject. How much of what we call cancer in the general population can be attributed to exposure of this type? A brief answer would suffice, even a guess at a number. I've heard numbers up to 90% of all cancers attributed to environmental factors.

DR. TORKELSON (Dow Chemical Company): Will you define environmental first?

DR. COURI: Yes, there are two kinds of environment, one is a workplace and the other is a public environment where people live.

DR. TORKELSON: Are you talking about the workplace or are you talking of the environment in general?

DR. COURI: Both. They are two separate types of environment.

DR. TORKELSON: Then you have asked two specific questions.

DR. VanPEENEN (Dow Chemical Company): I'd like to comment on this issue because I think Dr. Torkelson's point is very crucial. There was some discussion on this point several years ago by Dr. Higginson who spoke about international variation in risk of death from specific cancer types. He believed that approximately 80-90% of all cancers could be attributed to environmental factors. He thought if you looked at cancer data of good quality and completeness from all countries that there would be some place when the rates would be the lowest and that the lowest rate was of genetic origin. This implied that there was some normal genetic rate of cancerous mutations for man similar to other species and strains of animals. He felt that the remaining 80-90% of cancers were related to environmental causes and that this number might be capable of reduction. I wouldn't go that far because I know that experiments on animals have shown that this genetic versus environmental factor is a very artificial one depending on the definition. In the case of yellow shanks in chickens, if you take a purebred genetic strain of chicken that is susceptible, you can produce yellow shanks by feeding them with yellow corn but not with white corn. To that extent, it's probable that this reasoning is justified that cancer is caused by the environment. Dr. Higginson has gone to great lengths to indicate that by environment he is also referring to things like lifestyle. We certainly know that many cancers in humans are known to be linked to several known causes such as cigarette smoking or ionizing radiation, and if

you talk about number of cancers rather than number of deaths, exposure to sunlight. As to what proportion of all cancers are caused by exposure to the workplace environment, that is probably a much smaller number. A government document was put out some time ago that raised estimates that about 40% of all cancer in this country could be caused by workplace exposure and as much as 18% from asbestos alone. That document has been one of the most heavily criticized documents in epidemiologic meetings and in schools of public health as a training exercise.

DR. ALI (Ohio State University School of Medicine): Dr. Norman, I would like to ask a question about your study on tetrachloroethane exposed workers. I think you used the term malignant and I would like to know how you justified the malignant neoplasia with this compound. Was there any specific tissue affinity and how many organs or systems were looked at? Can you give me some information about which system or which tissue had the greatest frequency of malignancy or neoplasia?

DR. NORMAN (National Academy of Sciences): As to your first question, this was a study based on death certificates and the death certificate listed causes of death were the basis for the diagnosis of malignancy. I do not know the percentage of those death certificates for malignancies that were actually confirmed by autopsy and histopathologic examination. Just on the basis of looking through some of them during the course of the analysis, I would expect between 30 and 50% had been autopsied. As to your second question, the tetrachloroethane group consisted of the people who actually were exposed or could have been exposed on the basis of their military occupational specialties. I found the largest risk to be for cancers of the genital organs, leukemia, aleukemia and other lymphatic cancers. The other lymphatic cancers included three cases of lymphosarcoma and one follicular cell lymphoma. These are the only tissues that showed any indication of an increased relative risk. As I pointed out in the paper, however, only the leukemia and aleukemia incidence were really strongly suggestive of a definite effect related to tetrachloroethane exposure.

DR. TORKELSON (Dow Chemical Company): Dr. Ali, were you speaking of chemical affinity?

DR. ALI (Ohio State University School of Medicine): Yes, I was asking about tissue affinity for the compound or the chemical to induce a neoplasia.

DR. TORKELSON: Tetrachloroethane is a typical lipid solvent. You would expect it to be taken into tissues that are fatty, but I think one has to be cautious about this point because 50% of most cells have a fatty nature. The cell walls and a lot of other parts of almost every cell are part lipid in nature.

DR. KAPLAN (SRI International): Dr. Norman referred to my results in his talk and I'm afraid my slide may have inadvertently misled him. I did not present any results for leukemia on that slide because of time and space considerations. The reason I didn't was that the results were not remarkable for all neoplasms of the lymphatic and hematopoietic system including leukemia. The risk was only approximately 80% of that of the comparable general population.

What was shown on the slide was for diseases of the blood and blood forming organs which had the highest SMR of any of the causes listed. That incidence was based on three deaths and when we looked at them in detail the diseases had nothing in common. One was an anemia, one was a thrombocytopenia, and one fell into a miscellaneous category.

DR. VanPEENEN (Dow Chemical Company): I mentioned earlier in my talk that Dr. Dougherty had asked me to discuss with this group the possible significance of a slight increase in bilirubin that was observed. I didn't mention that measurement in my talk, but in this particular study of methylene chloride workers, there was a statistically significant increase in bilirubin levels. Statistical significance and clinical significance are, of course, two different things. The difference between the means was 0.7 milligrams percent versus 0.5 which in an individual patient wouldn't mean anything. I wonder if anybody might want to comment on the biological significance of this increased mean bilirubin level in the exposed group? The bilirubin level was elevated in the absence of any indication of hemolysis or of a blood abnormality.

LCDR PITTS (Naval Medical Research Institute): Dr. VanPeenen, was this a direct bilirubin or a total bilirubin determination?

DR. VanPEENEN: It was direct bilirubin.

LCDR PITTS: The only causes of an increased direct bilirubin level that I know of are either some type of active hemolysis or liver blockage.

DR. WEN (Gulf Science and Technology Company): One area that has not been really looked into is laboratory variability, either day to day, or from lab to lab or from year to year. When you're analyzing large sets of data, the quality of the lab becomes a major issue in calculating your statistical significance and may account for the differences that you observed.

DR. MASTROMATTEO (Inco Limited): My question is directed to Dr. Kaplan. I know you addressed some of the problems of using union records to go back and do an epidemiologic study but I wonder if you would comment further on that problem. For example, in union agreements, if a person leaves his employment, does he also leave the union? Do you lose track of a lot more people by working through union records? I also wasn't too sure from your description whether all of the study subjects were exposed only to perchloroethylene or other solvents such as a stoddard solvent as well. I think you did discuss it, but I wasn't sure at the end how you could be certain that this cohort was exposed to perchloroethylene and nothing else.

DR. KAPLAN (SRI International): The first question was about the union records and, in general, I think your point is well taken. In these particular union locals they retained the records of their former members. The second question was about exposure to other solvents. The study was intended to evaluate exposure to perchloroethylene and that was a criterion in our contract. There are obvious reasons to exclude people with mixed exposures to carbon-tetrachloride and trichloroethylene but in practice trichloroethylene did not have much use in the dry cleaning industry because it makes dyes run and is very harsh on synthetic fibers. There were probably only about ten shops in the

country that used trichloroethylene and then only for a brief time. Carbon-tetrachloride was important early on and we did eliminate that. We were concerned about perchloroethylene rather than the effects of the petroleum solvents and we did not exclude those who otherwise qualified who were also exposed to petroleum solvents, including stoddard solvent. I suspect that if we had only included those we knew were exposed solely to perchloroethylene we would have studied as few as five individuals.

DR. TORKELSON (Dow Chemical Company): I have a question about a point that's bothered me, Dr. Kaplan. You looked at the dry cleaner population and immediately seem to say that whatever was found was due to the solvent exposure. Have we ruled out the effects from other chemicals that might be used for spot removal, heat stress and exposure to human dander or fabric dust? We seem to quickly jump on the solvent as a cause of injury because it's a nice volatile material and guilty until proven innocent.

DR. KAPLAN (SRI International): I don't believe that I implied in my presentation this morning that the findings were due to perchloroethylene or other specific materials being ruled out as a cause. All we are saying is that we have studied a population of individuals, dry cleaners, with known perchloroethylene exposure that occurred long enough before the study for cancers to occur if they were an occupational response. I would concur very strongly with some of the earlier remarks made by Dr. Wen about the difficulties we have in differentiating between mixed exposures in epidemiologic studies because where you study people with specific chemical exposures, they are almost always occupational. It's very difficult to rule out other possible explanations such as the kind that Dr. Torkelson mentioned.

MR. JONMAIRE (Uniroyal, Incorporated): Dr. Wen, when you looked at your data and you saw that atherosclerosis was lower in the exposed group versus the control population, how did you explain it?

DR. WEN (Gulf Science and Technology Company): Generally these findings are attributed to the so-called "healthy worker effect". When the work force was employed, there was a selection factor. They were selected as healthier than the general population as a condition of employment. After hiring there is an improvement in socio-economic status and mental well-being. These are all factors that appear to contribute to the stability of a family structure, the stability of psychological status, and provide a better environment for the employee which may result in lower risk for coronary heart disease. It is my belief that this "healthy worker effect" is a combination of selection factors by the company as well as an improvement in the environment after the employment.

DR. LEFFINGWELL (National Institute for Occupational Safety and Health): The so-called "healthy worker effect" is not uniform across all causes of death. It is selective and I think you'd expect to see a particularly prominent depression in cardiovascular diseases in the situation Dr. Wen was dealing with. Perhaps an environment like this where smoking is prohibited because of an explosion hazard might be a better and healthier place to work.

DR. KAPLAN (SRI International): I would like to elaborate on Dr. Leffingwell's point about the "healthy worker effect" not being uniform because now we have more than speculation. There are some data from Great Britain where surveys comparing the general population with the employed population have been conducted which have shown that the "healthy worker effect" is very different for the entire population. It's most striking in the reduction of respiratory and cardiovascular diseases.

MR. JONMAIRE (Uniroyal, Incorporated): If workmen don't die of cardiovascular or respiratory diseases, don't they have to have a higher incidence of something else?

DR. LEFFINGWELL (National Institute for Occupational Safety and Health): I hope not to be too technical here. The way in which the SMR takes into account, eventually people have to die of something. In the long run, that may very well be true. What we are talking about here is technically called competitive risk. People have been looking at the question of reduction in risk of death from diseases of the cardiovascular system, particularly myocardial infarction with its possibility of resulting in an increased incidence of cancer death. Based on modeling and fitting the data, the number of additional survivors, and their ages, it seems like the impact is minimal.



SESSION V

AVAILABLE EVIDENCE FOR CONSTITUTIONAL  
HYPERSENSITIVITY TO ENVIRONMENTAL AGENTS

Chairwoman

Hoda A. Guirgis, Ph.D.  
Associate Professor  
Department of Community and  
Environmental Medicine  
University of California, Irvine  
Irvine, California

APPROACHES TO THE GENETIC STUDY OF  
ENVIRONMENTAL (CHEMICAL) CARCINOGENESIS

Hans Meier, Ph.D.

Senior Staff Scientist  
Jackson Laboratories  
Bar Harbor, Maine

The work I'm going to outline briefly for you this morning is part of a comprehensive multi-disciplinary study in inbred strains of mice and the rabbit aimed at identifying and mapping genes which confer either increased or decreased susceptibility to tumor development, i.e., both spontaneous and induced tumor developments. Clearly, the individual susceptibility differences in tumor development should lend themselves to a very detailed genetic analysis. Through the use of inbred strains, appropriate strain crosses, primarily back-crosses, mutant and congenic mice as well as particularly recombinant inbred (RI) strains, we wish to establish phenotypic differences to a variety of carcinogenic chemical classes, pathologically, biochemically, virologically and immunologically. We are employing dose responsiveness, using all possible routes including transplacental application of drugs, and the carcinogenesis indices as defined by Iball, to make the comparisons among the strains and strain crosses. Pathologically, there is very little succinct evidence available on the factors responsible for the histologic type of tumor eventually produced, be it spontaneously occurring or upon induction by chemical carcinogens. Biochemically we are using a variety of enzyme markers, drug metabolizing enzymes, etc. in trying to establish metabolic profiles. Virologically, we have been concerned for the last 15 years with the host genetic regulation of both ecotropic endogenous murine leukemia viruses as well as the xenotropic endogenous murine leukemia virus. Clearly, there are interactions between the chemicals that we are applying and the endogenous virogenes. Immunologically, we are using cell surface markers primarily for studies that may be linked to genes that have a bearing on susceptibility to cancer induction. The H-2 complex is a cell surface marker of which there are some dozen or more alleles known, each with different specificities. The H-2<sup>oz</sup> is an intra-H-2 recombinant which we have found containing specificities of both the H-2<sup>b</sup> allele and the H-2<sup>k</sup> allele. Nude streaker is an athymic mouse which we are employing to understand what role T lymphocytes may play in the induction of cancer.

As far as dose-responsiveness is concerned, we are employing maximally tumor-effective doses after establishing a dose response curve. The carcinogenesis index is the percent tumor induction divided by the latency period of tumor development in days times 100. After establishing phenotypic differences which we then can subject to genetic analyses, we look for linkage relationships (obviously there are major and minor genes which have a bearing on

both induced and spontaneous tumor development) and we can also look for gene interactions; for example, interaction between the alleles at the Ah locus of which there are several alleles known, with the endogenous viro-genes, with H-2, the cell surface marker and its specificities. In addition, we can look for allelic effects, particularly at several loci including Ah. There are a number of alleles now known at the Agouti locus, all of which we have placed on a C57BL/6 background so we don't have influences on the expression of these genes by the genetic background. H-2 is another very important one; we have already found that H-2 very significantly associates with certain tumor types and also with tumor progression.

One of our major efforts relates to marker genes on chromosome 15. Chromosome 15 is important for at least two reasons; one is that if one induces thymic leukemias in mice, be it by chemical carcinogens, by oncogenic viruses or by radiation, chromosome 15 is always trisomic indicating there is something very specific about that chromosome. Chromosome 15 contains some 20 or 22 known genes, two of which are polymorphic genes. The first one is the cytoplasmic form of glycerophosphate dehydrogenase (Gdg-1) and the second one is the glutamic-pyruvic transaminase (Gpt-1) gene of which there are two alleles known, while the Gdc-1 locus has three known alleles. The second point of interest here is that recently King and Lynch and others have found a gene in man which is closely linked to the GPT locus that confers susceptibility to the development of breast cancer in cancer families. So we are particularly focusing on chromosome 15 in the mouse; by the way, the GPT gene in man is assigned to chromosome 10, but that's semantics.

We are also looking at a number of selective mutations which already have been found to be associated with certain tumor developments; for example, the steel (sl) gene is one that causes a lymphoproliferative disorder in certain WK mice and so is the W gene; these are all pleomorphic gene effects. A number of other genes have been found to be associated with leukemias; in particular, the dilute gene, the fuzzy gene, and the hairless gene in our own laboratory. I've talked about the Agouti gene and some of the other genes maybe rather vestigial genes but one of these in fact may be the Ah locus; who knows?

Eventually we wish to establish the biochemical basis and effects of these genetic analyses relevant to carcinogenesis, particularly the finding of new pleomorphic genes and we want to learn about the modes of gene action that may be important in carcinogenesis.

The known polymorphisms in the mouse are obviously of some importance in carcinogenesis, particularly at the Ah locus. I'm talking about a single Ah locus although there may be several loci. In order to explain certain of the results in chemical carcinogenesis experiments, one must assume at least the presence of six alleles. We have recently mapped and located the gene for microsomal epoxide hydrolase to chromosome 1. The polymorphism here relates to the pH optima and heat denaturation kinetics. The two strains that differ are the BL/6 to which we assign the b allele and the DBA/2 strain to which we assign the d allele. This is an enzyme which is different from the cytosolic form and the in vivo significance of this polymorphism is not as yet known. Interestingly, it may be paralagous to the coumarin hydroxylase locus which

we have also located and mapped to chromosome 7. The suggestion has been made that chromosome 1 and chromosome 7 may have derived by tetraploidization from a common ancestral chromosome. Recently, Wendell Webber at the University of Michigan has found significant differences in the ability of certain strains to acetylate carcinogenic aromatic amines; for example, amino-fluorene and this locus has not been mapped as yet and therefore has no gene symbol. Ira Gurtoo has found another locus that seems to code for the aflatoxin B-1 4-hydroxylase. This would not be an activating enzyme, but rather a degrading enzyme which he feels may be different from but perhaps linked to the Ah locus and maybe under the same control since the Ah locus controls a variety of mixed function oxidases. Again, strains BL/6 and DBA/2 differ.

The epoxide hydrolase locus (Eph-1) which I just described has been mapped in our laboratory to chromosome 1 some 25 centimorgans from the dipeptidase (Dip-1) locus. This is of interest because we have found in previous studies that there is a gene or a set of genes mapping to Dip-1 which is very much associated with the development of reticulum cell sarcomas in some of the BL/6, BL/10, and C57L strains of mice. Also, we have found closely linked to the Eph-1 gene the gene that codes for the inducibility of the xenotropic endogenous murine leukemia virus which we call Bxv-1. Whether or not that is also the structural gene for the endogenous xenotropic virus, we don't know as yet.

Similarly in the rabbit there are a number of polymorphic loci known which we can use advantageously for marker studies. For example, the esterase -1 locus of which there are two alleles known, the esterase -2 locus of which there seem to be four alleles that code for cocaine esterases or atropine esterases, respectively. There is a third locus for which in fact all the rabbit strains that we have at the Jackson Laboratory are identical; they carry the d allele uniformly. And these genes code for the ability to hydrolyze esterified drugs. VanZutphen, a former collaborator of ours, has identified a number of phenotypes with regard to hydrolytic activity and identified the respective genotypes. As in the mouse, rabbit strains differ in their capacity to acetylate certain drugs. There are some rapid and slow acetylators. Some of these loci we can utilize for marker studies as we do in the mouse. The Est-1 and the Est-2 loci may or may not be identical. I say this because we have not found any recombinants between them as yet. But on the basis of substrate specificity they do seem to be different.

The linkage map of the rabbit is of course by far less advanced than that of the mouse. There are still a lot of uncertainties, but linkage group 6 contains the esterase loci that I've referred to previously. There is a distance estimate of 6.3 centimorgans between Est-1 and Est-2. How accurate this is, I don't know, except as I said, we have not found any recombinants between the two loci.

Quite a while ago we undertook to study the host genetic control of endogenous retro-virus expression in mice, and in order to do that, we made all possible crosses between strain AKR and strain C57L. These two strains differ with respect to the expression of these endogenous viruses. The AKR strain is characterized virtually throughout their life by expression of ecotropic

murine leukemia virus whereas the C57L never expresses ecotropic endogenous murine leukemia virus. So in making all possible crosses, including reciprocal crosses, we have found that there are two genes segregating for the expression of endogenous virus. They are now termed Akv-1 and Akv-2 and their chromosomal locations have been mapped. In order to study what consequences may be with regard to tumor development, we have splenectomized these backcrossed mice at three or four weeks of age and assayed the spleens for their expression of ecotropic murine leukemia virus and then set the mice aside for tumor development. After having established that splenectomy does not influence the occurrence of most of the tumors in these two strains, what we found is that all of the mice that expressed high titers of either virus or the group-specific antigen (the P30 antigen of these viruses) early in life in their spleen were those mice that preferentially developed tumors later on in life. So the occurrence of these viruses or their antigen early in life is an important determinant and predictor, in fact, of the development of tumors later on in life. Only 14 tumors developed in originally virus-negative mice so one can conclude from this experiment that the presence of expressed viro-genes inherent to the AKR mouse is a very important predictor of the risk for developing tumors.

I've mentioned that the AKR mouse is a high leukemic mouse. It develops thymic leukemias to nearly 100% at one year of age. The C57L strain is a low tumor strain, developing reticulum cell sarcomas when they're something like two or three years of age. Interestingly enough, when we made crosses between the two strains, a whole set of other tumors and tumor types occurred, clearly indicating that there are additional genes contributed by both strains that have a bearing on the development of these types of tumors. So one can say that it is unlikely that tumor development is due to a single gene although a single gene may have major effects but rather that tumorigenesis is an expression of the contributions of a variety of genes.

The additional set of genes that we postulate to occur in this cross, the C57L/AKR cross, is being analyzed with the help of recombinant inbred strains. Those that we have developed are referred to as AKXL-RI lines; they are developed principally by crossing two strains that differ with regard to some of the traits that you want to study and F2s are made and the F2 is inbred for as many generations as possible. Then they can be typed for a variety of marker genes. Of the 4 sets of RI strains, we have a number of lines within them; as many as 26 in the case of the BXH and BXD strains, and, clearly, the more strains you have developed from the F2, the greater is the strain distribution pattern for any one gene under investigation. The rationale for RI strains is this, that by the F2, the genes which are identical in the two strains are fixed; whereas the genes which differ can assort and recombine independently of one another in different constellations. So we are typing these mice for as many markers as we have systems to type them for, and I can say that in the case of the BXH-BXD lines, we have them typed for as many as 100 loci. These loci are distributed over as many as 16 chromosomes where each chromosome contains at least one of the markers for which we typed them. So this is a very powerful new tool for genetic analyses, particularly of very complicated traits such as cancer obviously is.

We have recently discovered an example of a possible polymorphism in collaboration with Dr. Sloane at the University of Tennessee. We have analyzed the tumorigenicity of certain benzpyrene derivatives, the 6-formyl compound and the 6-hydroxymethyl compound and applied them to mouse strains C3H/He, DBA2, and their F1. This is a test that very rapidly gives you an indication of whether a gene, if segregating, is dominant or recessive. Assuming that the 6-hydroxymethylbenzpyrene compound (which is the least tumorigenic, producing only a few tumors in the C3H strain only) has to be converted to the 6-formyl compound in order to be effective, one can say that perhaps the DBA2 and the F1 lack a gene which allows dehydrogenation of this compound to the formyl compound. This is a polymorphism that is still under study. Where this gene may be, we don't know as yet. The difference in the tumorigenicity of benzpyrene among these three strains relates to the Ah locus. The DBA2 strain carries the d allele at the Ah locus which causes it to be resistant to the induction of tumors by benzpyrene, whereas the C3H and the F1 carry the b allele which is the allele for inducibility of arylhydrocarbon hydroxylase at this locus. So we clearly can distinguish between two different genetic systems that may be operative in this case.

We have done some induction studies for arylhydrocarbon hydroxylase in the rabbit and there are clear strain differences. Strain III is a relatively highly inducible strain relative to the wire-hair strain which is hardly inducible at all. But I must say that the magnitude of the inducibility is much, much lower than in the strains of mice that we have analyzed. This may explain the fact that rabbits are exceedingly resistant to the induction of tumors by polycyclic aromatic hydrocarbons. We have made a number of crosses between the most inducible and the least inducible strain and find that the genetics are much more complex than that of the mouse. The induction here, even in the more susceptible strain III, is maybe three-fold, at the most, whereas in inducible strains of mice such as the C57BL/6 mouse, it may be as much as twenty-fold.

There is a combined pedigree of two rabbit strains, wire-hair and strain 10 with a possible site of a mutation which causes lymphosarcoma to occur in the wire-hair strain and autoimmune hemolytic anemia in the strain 10. A recessive mutation occurred which confers susceptibility to spontaneous lymphosarcoma development in the wire-hair strain which we have termed ls and in strain 10, autoimmune hemolytic anemia which we termed ha. Since these two strains are related, we wonder whether in fact the two presumed mutations, ls and ha, may be identical but have a different phenotypic expression on account of the interaction with the genetic background that is somewhat different between the two strains. And, indeed, when we made the respective crosses, it came out that double heterozygotes developed the conditions in the appropriate Mendelian ratios of 1:4. So we now termed this gene ha simply because the autoimmune hemolytic anemia phenotype occurs earlier than that for lymphosarcoma development. Here then we have a situation where the same gene can give you a totally different phenotype depending on the genetic background.

There exists a condition referred to as renal cortical cyst formation in certain of the wire-haired strain derivatives. Cortical cysts are caused by a recessive gene referred to as rc which is similar to the simple cysts that occur in man. We found that in cases where this renal cyst formation gene segregates, that these rabbits are much more susceptible to the development of tumors following ethylnitrosourea (ENU) application, and, strangely enough, the tumors seem to begin with these cysts. Eventually you end up with a kidney that looks like a Wilms tumor or a nephroblastoma involving virtually the entire kidney. In situations that we created where fetuses are presumably heterozygous for the ls or ha gene, very often we also get a mimicry of an ls homozygote indicating that the transplacentally administered ENU caused a phenotypic change of the heterozygote to homozygote and this is the subject of a current investigation. This is a very interesting situation that has been found with regard to the Brachyuri gene in mice when they were treated transplacentally with 5-fluorouracil.

When we make crosses between strain IIIIVO and the strain WH (wire-hair) that does not have the rc gene segregating in it, we find that a transplacental administration of ENU causes the occurrence of a high incidence of tumors, independent of which way we made the cross. This is a characteristic of the IIIIVO strain whereas the wire-hair strain without the rc gene gives you a low incidence of ENU-induced renal tumors. We can say from this simple experiment there must be at least one autosomal dominant gene present in the strain IIIIVO which confers susceptibility to ENU-induced tumors. There may be more than one gene involved. In addition, there are a number of questions still open. For example, why is the kidney the target organ of the ENU and what is the reason for the resistance of the wire-hair strain?

Interestingly enough, exclusively in the strain IIIIVO we find in addition to transplacentally induced renal tumors neurofibromas or schwannomas involving the peripheral nerves. This is also subject to genetic analysis and we find that possibly just one or a few genes (autosomal dominant genes) may be involved in the pathogenesis of these nervous system tumors. On occasion we find subneuralemmal cysts associated with these peripheral schwannomas.

When we administer ENU on a chronic low dose basis to pregnant rabbits, we find that many of the embryos are miniaturized. The important thing about this teratologic effect is that many of these miniaturized animals do survive so that they may afford us the possibility of analyzing the relationship, if any, between teratogenesis and carcinogenesis. This is a field that's fairly open and unclear as yet although many carcinogens are also teratogens. Mostly those animals (miniaturized animals) that do die have holes in their parietal bones, a lesion that is incompatible with survival.

Similarly in mice that are treated transplacentally with ENU at eight to ten days of gestation and the fetuses harvested at day 14, one sees a variety of skeletal abnormalities, e.g., absent metacarpals and metatarsals. You see wavy and fused ribs, lack of tail, etc. Aside from these skeletal abnormalities you may also see a variety of soft-tissue abnormalities such as spina bifida, anencephaly, exencephaly, open eyes and so on.

When we have attempted to analyze the differences in the effects of ENU given transplacentally to mice, we can clearly see that there are genotype-dependent effects. The total of the dead, resorbed and malformed fetuses suggest that apparently this trait is due to a number of genes. It has a multi-factorial inheritance. When we make crosses between the most resistant and the more susceptible strains (again, the AKR and the C57L) we find that all those mice in which endogenous ecotropic viruses are expressed are resistant to the teratogenic effects of ENU.

I just want to caution that crosses always have to be made reciprocally because when we induced lung tumors and leukemias in reciprocal crosses of the same strains, we found that where the SWR mouse is the mother, the occurrence and incidence of tumors is much greater and occurs earlier than when the AKR is the mother.

To conclude, what I have tried to indicate today is that cancer development is at least in part controlled by host genes and probably there are a number of genes involved in oncogenesis. Obviously, carcinogenesis is a very complex phenomenon, but we do know that certain single genes can exert major influences. But it's unlikely that single gene determination is the major cause of a carcinogenic process.

We must identify and characterize these genes and primarily find out their mode of action. Thus, our program is really not aimed at identifying new carcinogens or mutagens, but rather to find out the extent to which the carcinogenic potency is host-gene dependent. I've alluded to the fact that many of the linkages (in the mouse or in man) are evolutionarily conserved so it doesn't matter whether the GPT gene is on chromosome 15 in the mouse versus on chromosome 10 in man. The linkage relationships are generally preserved.



PAPER NO. 29

STATUS OF ARYLHYDROCARBON HYDROXYLASE LOCUS AS A  
DETERMINANT OF CANCER SUSCEPTIBILITY

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Text of this presentation is not available for publication.

PAPER NO. 30

HEREDITARY DIFFERENCES IN THE HYDROLYSIS OF  
ENVIRONMENTAL CHEMICALS BY TISSUE AND SERUM ESTERASES

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Text of this presentation is not available for publication.

IS PULMONARY DYSFUNCTION A RISK FACTOR FOR  
MULTIPLE DISORDERS?\*

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In 1971, almost 10 years ago when a Specialized Center of Research (SCOR) in lung disease was established at Johns Hopkins, a study of genetic and environmental factors in chronic obstructive pulmonary disease (COPD) was initiated under its aegis. Necessarily comprising a multidisciplinary project because of its broad scope, the data derived from the joint efforts of our investigative team which includes Drs. Solbert Permutt, Harold Menkes, Earl Diamond, David Levy, Mary Meyer, Terri Beaty, Carol Newill and many others as well as myself; and accordingly the project draws upon the Departments of Medicine, Environmental Health Sciences, Biochemistry, Biostatistics, Behavioral Sciences, etc. in addition to the Epidemiology Department. What has turned out to be the most intriguing part of the findings is that in exploring risk factors for COPD not only does it appear that COPD is a prototype for multifactorial disorder, a disorder in which many environmental and genetic factors contribute to the risk of its occurrence, but more provocatively a hypothesis has emerged which extends beyond the causation of pulmonary obstruction to its effects: a hypothesis which has implications well beyond the obstructive pulmonary disease to other types of pathologic manifestations and to organ systems other than respiratory. But let us first consider risk for COPD, i.e. the risk of airways obstruction (Cohen, 1980).

The studies were conceived in a climate where a voluminous literature dating back to the mid-19th century had accumulated but many questions still remained unresolved. In the 60's, less than 10 years before the SCOR project began, alpha<sub>1</sub>-antitrypsin deficiency had promised to provide the key to emphysema. However, it was soon recognized that truly alpha<sub>1</sub>-antitrypsin deficient individuals (i.e. those whom we call "Pi Z" - protease inhibitor type "Z" individuals - persons who are homozygous for the "Z" allele or the "null" allele) are very rare in the population with a frequency of about 1 in 1500 in most Caucasian populations and still lower in others. And the findings as to pulmonary impairment among those heterozygous for "Z" and/or having other Pi variants have been equivocal, showing deviations from normal in some studies but not in others. Thus, in view of the rarity of truly alpha<sub>1</sub>-antitrypsin deficient individuals, coupled with the questionable finding for heterozygotes

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\* Supported by grant #HL 14153 from the National Institutes of Health.

and other Pi-variant types, it became apparent that this genetically determined marker probably accounts for very little of the observed COPD in the total population. Consequently, we undertook to examine the role, not just of  $\alpha_1$ -antitrypsin, but of a wide range of possible underlying causal forces for COPD (Cohen et al., 1975; Cohen et al., 1977). I won't pause to discuss the basic study population here except to note that for probing potential risk factors, a sample of 2539 adults, excluding all subjects ascertained as patients, was used. That is, only subjects not selected on the basis of their own health status are included in the analyses we'll be considering here.

To avoid the problems of unintentional bias in the clinical diagnosis of COPD, FEV<sub>1</sub>% (forced expiratory volume in one second expressed as a percentage of forced vital capacity) has been taken as an objective indicator of airways obstruction and thus of incipient, potential, or actual COPD. The indices used are mean FEV<sub>1</sub>% and rate of impairment (with impairment defined as FEV<sub>1</sub> less than 68% of FVC). For simplicity, only impairment rates are presented in most of the figures, although both mean FEV<sub>1</sub>% and rates of impairment have been calculated and display similar patterns.

A large battery of data was collected on the study participants: pulmonary function tests, blood and saliva studies, and a detailed interview regarding demographic, medical, family and other epidemiologic information as well as other tests and record data (Cohen et al., 1975). Thus far, more than 20 variables have been examined as potential risk factors or confounding factors. In addition to the basic epidemiologic variables - of age, sex and race - environmental agents such as cigarette smoking, socio-economic status (SES), coffee, tea, alcohol, diet soda and medications intake, as well as genetically determined factors such as Pi ( $\alpha_1$ -antitrypsin type), ABO blood group, Rh type, ABH secretor ability, PTC taste ability, and amylase type were studied. Also examined were mixed variables which may involve shared genes and/or shared environmental agents, such as height, medical history, and simply the familial aggregation of pulmonary dysfunction. The effect of each factor was computed taking into account the others so that its impact as given in the figures is an adjusted value, i.e. adjusted for other factors in a matrix of important variables. For most, an adjustment matrix of 14 or so variables was used, about the limit that our computer programs could handle simultaneously. A modification of the binary variable multiple regression method of Feldstein was the procedure used for adjustment (Feldstein, 1966).

Now, let's consider some of the factors. As in many chronic disorders, age and cigarette smoking appear to be the major risk factors (Table 1). Being of low socio-economic status, being a first degree relative of a COPD patient, being male, all seem to show significant increments in pulmonary dysfunction, while black-white racial differences did not show a consistent relationship to impaired pulmonary function.

As for genetic markers, in addition to carrying at least one Z allele in the Pi ( $\alpha_1$ -antitrypsin) system, having an A blood group antigen in the ABO system or being an ABH non-secretor, especially in whites, appeared to be associated with poor pulmonary function, while PTC taste ability showed no association.

TABLE 1. RISK FACTORS AND ADJUSTED FEV<sub>1</sub>% IN ADULTS EXCLUDING PATIENTS

(Each variable adjusted for all other variables - age, sex, race, smoking, socioeconomic status, Pi, familial component, ABO, secretor, PTC, alcohol, coffee, and tea)

N = 2539			
	No.	Impairment rate/100	Significance t
AGE			
20 - 29	469	3.5	...
30 - 39	465	4.4	NS
40 - 49	557	12.0	.0001
50 - 59	778	21.4	.0001
60 +	270	26.0	.0001
SEX			
Male	1295	16.1	...
Female	1244	10.7	.001
RACE			
White	1787	13.5	...
Black	752	13.3	NS
CIGARETTE SMOKING			
2+ packs/day	181	21.7	.0001
1 - 2 packs/day	598	19.4	.0001
Never smokers	719	7.0	...
Other	1041	13.0	.001
SOCIOECONOMIC STATUS			
Low	653	17.3	.0001
High	367	7.5	...
Other and unclassified	1519	13.2	.01
Pi (alpha <sub>1</sub> -antitrypsin) TYPE			
Z + Z carriers	182	18.5	.02
Other - variant	271	14.5	NS
αM	471	15.6	.05
Non αM	1615	12.1	...
FAMILIAL COMPONENT			
1° relatives of COPD patients	363	19.3	...
1° relatives of lung cancer patients	118	24.2	NS
1° relatives of nonpulmonary patients	245	12.9	.05
Other #	1813	11.6	.001
ABO BLOOD TYPE			
With A	1029	15.3	...
Without A	1491	12.3	.05
Not typed	19	1.3	~
SECRETOR			
Nonsecretor	372	17.0	.05
Secretor	1068	12.6	...
Unknown	1099	13.0	NS
PHENYLTHIOCARBAMINE (PTC) TASTE ABILITY			
Nontaster	644	13.0	...
Taster	1729	13.4	NS
Other	166	15.7	NS
ALCOHOL			
Light intake	1360	14.1	...
Moderate intake	432	11.1	~
Heavy intake	727	13.7	NS
Other and unknown	20	9.9	NS
COFFEE			
3+ cups per day	966	15.5	...
None	508	10.8	.02
Other and unknown	1065	12.8	~
TEA			
3+ cups per day	369	15.1	...
None	369	15.1	...
Other and unknown	451	15.7	NS
	1719	12.5	NS

Impairment = FEV<sub>1</sub> < 68% FVC

# = Other, includes nonpatients in categories other than specified, as well as unknown or unclassified.

NS = Not significant.

... = Reference category for t

~ = Borderline

Also examined were the rates of impaired pulmonary function in relation to coffee, tea, and alcohol consumption (Cohen et al., 1980). Although it was not surprising that coffee intake, but not tea, appeared related to pulmonary impairment, the absence of an association of alcohol consumption was unexpected in view of the extensive literature associating alcoholism with an increased prevalence of not only tuberculosis but also other pulmonary problems. While we cannot rule out a role for alcohol in the development of pulmonary disease, its impact, if any, is probably the result of interaction with other intrinsic or extrinsic factors such as smoking associated with alcoholism, if not deriving primarily from those other factors (Cohen et al., 1980).

Thus, in all, a sizable list of potential risk factors for airways obstruction has accrued, significant associations having been observed with age, being male, cigarette smoking, low socioeconomic status, heavy coffee intake, and such genetically determined traits as Pi Z, A blood type, ABH non-secretor status, as well as being a relative of a pulmonary patient, having a history of asthma, and taking of allergy medications. Whereas some variables appear more important than others, no one factor, yet identified, seems either necessary or sufficient to produce COPD, or to be singularly associated with COPD. Doubtless some other factors influencing airways obstruction are yet to be uncovered; and combinations of factors and their interactions need to be explored.

While identifying the multiple risk factors associated with COPD has provided a stimulating challenge, the direction that has become even more fascinating was suggested during further study of the familial aggregation data. Now, certainly, familial clustering per se would not be a candidate for a distinguishing feature in any chronic disease. What was intriguing, however, was that first degree relatives of lung cancer patients, like first degree relatives of COPD patients, had significantly higher rates of impaired pulmonary function than the corresponding relatives of non-pulmonary patients or the neighborhood controls, teachers, or other nonpatients. And, as with relatives of COPD patients, this excess of impairment in relatives of lung cancer cases could not be accounted for by any of the adjustment factors: smoking, age, sex, race, socio-economic status, etc. (Cohen et al., 1977). Also noteworthy is that these observations which we have reported previously based on a smaller sample and adjusted for fewer confounding variables have persisted in the larger series and with adjustment for additional factors. Moreover, the pattern was consistent even in tabulations limited to those who had never smoked cigarettes. Thus, it appears that lung cancer and COPD share a common familial pathogenetic component associated with pulmonary dysfunction but not entirely accounted for by smoking.

However inviting this observation may be for tracking down shared genes and shared environmental agents, it is even more provocative that in two diseases with such different pathology as lung cancer and COPD, there is a similar pathophysiological characteristic, impairment of pulmonary function. Emphasizing this finding is the accumulating evidence from other investigators suggesting that airways obstruction irrespective of cigarette smoking is associated with an increased risk of neoplasms of the lung and bronchus (Davis, 1976). Yet, in spite of the fact that the similar manifestations of pulmonary impairment among the relatives of lung cancer and COPD patients cannot be attributed to cigarette smoking, the relationship of cigarette smoking both to impaired pulmonary function and to these two diseases is clearly established (U. S. Department of

Health, Education and Welfare, 1979). Also well documented is the association between cigarette smoking and increased risk of such pathologically diverse conditions as coronary artery disease, bladder cancer, hypertension, lymphomas, etc. as well as with lung cancer and COPD (Kahn, 1966).

Moreover, these elevated mortality ratios of regular smokers reported by Dorn over 20 years ago have been supported by subsequent mortality studies, by morbidity and disability data, and by animal studies. Even though the statistical significance of the associations between smoking and a wide range of diseases is well recognized, nevertheless, the multiplicity, diversity and putative nonspecificity of the effects of cigarette smoking have long been a source of puzzlement to epidemiologists. Such a quandary need not exist any longer, however, if one accepts the hypothesis that impaired pulmonary function caused by smoking can lead to a broad spectrum of manifestations, not only in the airways, but also in the circulation and vasculature, and, in fact, in the tissues of all organ systems (Cohen, 1978). Simply, then, is pulmonary dysfunction the common denominator for the multiple effects of cigarette smoking?

But before considering the rationale for this hypothesis, let us make one slight modification. Because impaired pulmonary function is postulated to be the underlying mechanism inducing, precipitating, or potentiating, the many effects of smoking, let us generalize the hypothesis by substituting for cigarette smoking, any and all causes that will compromise pulmonary function (Cohen, 1980).

The more general question therefore is: Can impaired pulmonary function, whether caused by environmental agents, genetic predisposition or other constitutional susceptibility, or a combination of these - as is more likely - lead to multiple pathologic effects, affect all organ systems (not just respiratory); and thus be a risk factor for numerous clinical manifestations and syndromes? And, if so, how?

Let us examine this in a step-wise manner looking at the various individual relationships, separately, and filling in some of the details.

The first condition that needs to be considered is clinically manifest COPD. Impaired pulmonary function is pathognomic for clinical COPD - which in fact is defined by impairment of forced expiration. There may even be a self-accelerating effect of airways obstruction. For, pulmonary dysfunction may lead to increased accumulation of inhaled pollutants, particulates, and toxins, which in turn probably further accentuate the embarrassment of pulmonary function.

The observed association of lung cancer with pulmonary dysfunction in other studies, as well as our own, becomes entirely plausible when reviewing the apparent physiological consequences of impaired pulmonary function in man, in addition to the experimental animal evidence for induced lung cancer. Clearly, where pulmonary dysfunction has interfered with the effective elimination of noxious agents from the airways, there would be prolonged exposure to any carcinogens inhaled from external sources, doubtless enhancing the potential effectiveness of those carcinogens. Moreover, any tissue deterioration resulting from pulmonary dysfunction would mean that the carcinogens would be acting

on damaged, and thus primed, tissue. The importance of prior tissue injury in lung carcinogenesis has been indicated not only by the experimental animal studies but also by investigations suggesting that human bronchiogenic carcinoma tends to develop in already damaged lungs (Kuschner, 1968). (Think of uranium workers and the asbestos workers!)

Nor is the chain of events emanating from impaired pulmonary function limited to the respiratory system. The stagnation of inhaled substances in the lungs resulting from pulmonary impairment would increase the likelihood that excessive quantities of inhaled deleterious agents from extrinsic sources would enter the circulation. Meanwhile toxic metabolites and other wastes of intrinsic origin in the blood that are usually removed or metabolized by the lungs would not be effectively eliminated from the circulation in the presence of impaired lung function. Consequently, an excessive build up of harmful agents from both external and internal sources could result and could then lead to vascular damage reflected in the coronary, cerebral, or peripheral vessels, and in the clinical disorders associated with that damage.

Furthermore, the toxic waste burden of the circulation secondary to pulmonary inadequacy would be a potential hazard to all the organs and tissues of the body served by the blood and lymph vessels. Neoplasms of the bladder, prostate, uterus, and gastrointestinal tract as well as of the respiratory tract, lymphomas, ulcers of the stomach and duodenum, and cirrhosis of the liver are but a few of the conditions already noted to be more frequent in cigarette smokers. It may very well be that impaired pulmonary function from causes other than cigarette smoking may enhance the risk of these conditions - in some, directly, and, in some, by increasing susceptibility to, or exposure to, co-factors, precipitating agents, promoters or other key components. While the different manifestations of pulmonary dysfunction are probably not mutually exclusive, not every one develops every disease. Why?

Basically, there are differences in vulnerability among individuals, and in different tissues and organs within any individual - differences which derive from both genetic and acquired susceptibility of the tissues and organs as well as to the specific agents, carcinogens, allergens, drugs, hormones, etc. and to the pathogenetic processes involved: e.g., carcinogenesis, atherosclerosis, cirrhosis, etc. In addition, there is the variation in past exposure to deleterious agents of extrinsic and intrinsic origin as well as differences in previous medical history. Thus, given impaired pulmonary function, its ultimate effects depend upon numerous factors which include the competence of the body defense mechanisms to cope with exposure to various harmful agents, along with the type and degree of that exposure. Also, of course, where any condition ends in death, that would preclude the observation of any other effect in that individual. It is the familiar competing risk problem: if you die of one disease, you cannot get any other.

In conclusion, the impairment of such a fundamental mechanism as pulmonary function, whatever its cause, may precipitate a chain of events encompassing many types of tissues, organs, and pathology (Figure 1). However, only a relatively small proportion of the potential effects are ever manifested in any one individual, due to the dynamic interplay of genes, environment, and chance. Thus, while impaired pulmonary function may not be an absolutely essential



component for any single conditions, except for COPD (for which it is pathognomonic), nevertheless, it may act as a risk factor, initiator, promoter, or co-factor in the pathogenesis of numerous disorders (Figure 1).

**IMPAIRED PULMONARY FUNCTION**  
(IRRESPECTIVE OF ITS CAUSATION) CAN LEAD TO MANY DISORDERS

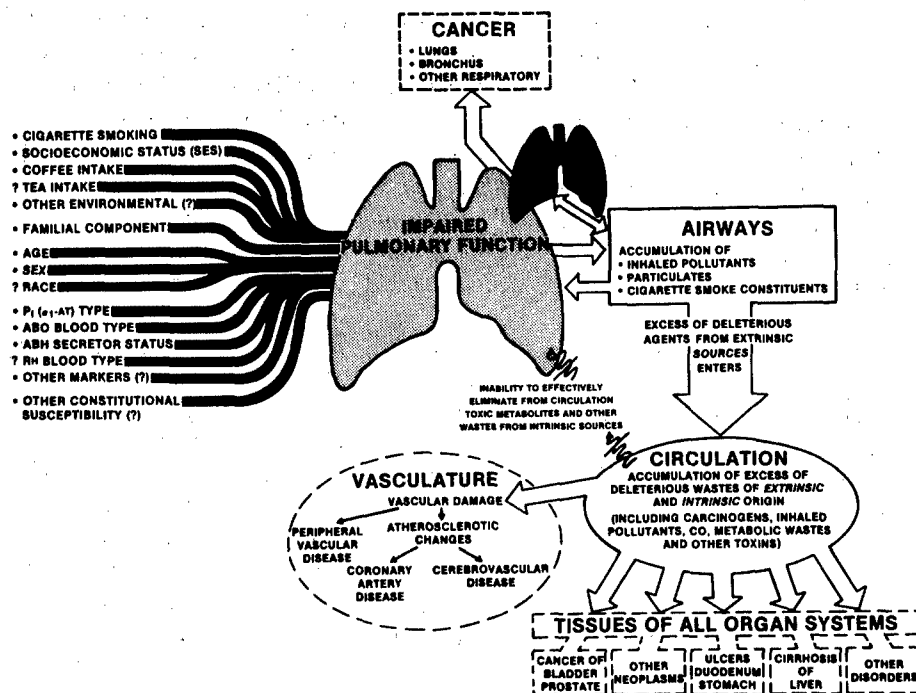


Figure 1. Impaired pulmonary function.

And so, as a mechanism influencing many diseases, impairment of pulmonary function could be termed a multi-disease risk factor, acting as a pathway to many and diverse pathological manifestations. Clearly these concepts of disease causation have broad implications not only in epidemiology but also in public health and clinical medicine. If, in fact, impairment of pulmonary function is a predisposing factor not only for disorders of the respiratory system but for a wide range of other conditions involving every organ system, the feasibility of using pulmonary function testing in the detection of risk for both respiratory and nonrespiratory disease should be considered. Moreover, in view of the broad based consequences of pulmonary impairment, concerted efforts to explore means of arresting and reversing deterioration of pulmonary function should receive greater emphasis in the research goals of preventive medicine and public health programs. Before any further speculation or consideration of implementation, however, the first step must be to test this hypothesis, preferably in large population samples and in multiple replications over different populations. In our SCOR (Specialized Center of Research) program at Hopkins, we are currently collecting longitudinal data not only to study further the

multiple risk factors in the causation of airways obstruction but also to explore its effects. Efforts to examine several different data sets have been initiated. We hope other investigators will also attempt to test this hypothesis, generating the necessary informative data from a variety of populations and using different approaches. In view of the implications of such causes and effects, we look to continuing research to obtain and analyze a variety of appropriate data - laboratory and epidemiological - to evaluate further the validity of the hypothesis I have just presented.

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#### ACKNOWLEDGEMENTS

The material presented here, including the table and figure, have been adapted from that published in Cohen, B. H., "Chronic obstructive pulmonary disease: a challenge in genetic epidemiology," American Journal of Epidemiology, Volume 112, number 2, August 1980.

The author also expresses her appreciation to Dr. Abraham M. Lilienfeld and Dr. Leon Gordis for their helpful suggestions as well as her colleagues already cited for their collaborative efforts in this project and her staff for their important contributions.

This work was supported in part by grant #HL 14153 from the National Institutes of Health.

IMMUNOBIOLOGIC ASPECTS OF PRIMATE  
HYPERSENSITIVITY PNEUMONITIS

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INTRODUCTION

Immunoregulatory defects are being reported in an increasing number of human disease states. These include decreased T suppressor cells in systemic lupus erythematosus (Sagawa and Abdou, 1978) and juvenile rheumatoid arthritis (Strelkaukas et al., 1978), associated with an antibody against a subset of regulatory T cells (Sagawa and Abdou, 1979). On the other hand, increased T cell-mediated suppression has been reported in common variable hypogammaglobulinemia (Waldmann et al., 1978), multiple myeloma (Hoover et al., 1981), selective IgA deficiency (Atwater and Tomasi, 1978), disseminated fungal diseases (Stobo et al., 1976), CLL (Faguet et al., 1979; Keller et al., 1979; Keller et al., 1979), various solid tumors (Zembala and Mytar, 1977; Yu et al., 1977), bone marrow aplasia (Hoffman et al., 1976; Abdou et al., 1978; Kagan et al., 1976), and burns (Miller and Baker, 1979). Of importance to this study, a recent report suggests that adherent suppressor cells are present in some patients with human tuberculosis (Ellner, 1975) and that FC $\gamma$  bearing T cells, a subset reported to be associated with suppressor or natural killer activity (Reinherz et al., 1980), are increased in the lungs of patients with active sarcoidosis (Hunninghake and Crystal, 1981). Although the circumstantial evidence of a role for suppressor T cells in disease pathogenesis and/or secondary immune defects is impressive, no study has rigorously demonstrated their role in human disease.

Hypersensitivity pneumonidities (HP) result from an inflammatory response of the lung initiated by the inhalation of organic environmental antigens. Despite their significant import for occupational medicine (Moore et al., 1974; Hansen and Penny, 1974; Fink et al., 1975), the etiologic factors involved in their pathogenesis remain poorly defined. One mechanism that has been implicated is immune complex deposition (Roberts and Moore, 1977). In pigeon breeder's disease, this hypothesis is strengthened by the finding of high titers of precipitating antibodies to pigeon antigens in the serum of patients (Fink et al., 1968). These antibodies are primarily IgG (Fink et al., 1969), although smaller quantities of IgM and IgA have been detected (Fink et al., 1967).

They are capable of binding complement (Boren et al., 1977; Caldwell et al., 1973) and binding to guinea pig tissue. Although their presence suggests a role for immune complexes in the pathogenesis of the disease, the findings (Fink et al., 1969; Boren et al., 1977; Caldwell et al., 1973) of similar titers of antibodies in exposed asymptomatic pigeon breeders raises a question regarding their involvement.

The chronic granulomatous inflammatory response observed in patients with hypersensitivity pneumonidities suggests a role for cell-mediated immunity in these disorders. Using peripheral blood, a number of groups (Hansen and Penny, 1974; Fink et al., 1975; Reynold et al., 1977) demonstrated that cells from a large percentage of symptomatic pigeon breeders produce migration inhibition factor on exposure to pigeon antigens. In addition, studies of antigen-induced blastogenesis (Hansen and Penny, 1974) further implicate circulating sensitized T lymphocytes. Furthermore, a recent study (Moore et al., 1980) reported a significant increase in the percentage of T cells in bronchial lavage fluid from patients with chronic HP. Another study (Flaherty et al., 1976) suggests there is a decrease in peripheral blood T cells in patients recovering from an episode of farmer's lung. Finally, symptomatic pigeon breeders have significant numbers of T cells in their bronchial lavage fluid which proliferate when stimulated with pigeon serum (Moore et al., 1980). Although a small percentage of asymptomatic patients also demonstrate migration inhibition factor-like activity in response to challenge, in vitro tests of cell-mediated immunity discriminate symptomatic from asymptomatic groups better than circulating antibodies (Hansen and Penny, 1974; Fink et al., 1975; Flaherty et al., 1976).

In order to better elucidate the mechanisms involved in HP, several animal models have been developed (Roberts and Moore, 1977). One model, developed in the stump-tailed macaque (Macaca arctoides) monkey, closely resembles human pigeon breeders in clinical presentation (Hensley et al., 1974). In addition, both humoral and cellular immune mechanisms (Hensley et al., 1974; Peterson, 1978) appear to be involved in the pathogenesis of monkey HP which is similar to the findings in human disease (Waldmann et al., 1978; Peterson, 1978). These observations and the phylogenetic proximity of monkey and man suggested that studies on the relationship of immunoregulatory imbalances in these groups of disorders could be performed using both patients and the animal model.

We have performed studies on eight symptomatic and eight asymptomatic pigeon breeders as defined by clinical history and the response to an intra-bronchial challenge to pigeon serum. These were compared to age and sex matched controls who were also matched for smoking habits. The patient groups had comparable exposure to their birds, similar titers of precipitating antibody in their serum, and normal pulmonary function tests (with one exception). None of the patients or controls were symptomatic with a viral illness or ingesting drugs known to alter immune function when studied.

In addition, we performed parallel in vitro and in vivo experiments on four stump-tailed monkeys using the model of Hensley (1974) for sensitization. After multiple intrabronchial challenges with pigeon serum, two of these monkeys developed classic signs of disease (fever, tachypnea, signs of pulmonic inflammation and leukocytosis) while two monkeys remained asymptomatic. We

therefore had both symptomatic and asymptomatic monkeys to determine the relationship, if any, of immunoregulatory elements to clinical disease in non-human primates.

Before discussing our results, it is important to briefly review a few techniques that were employed. The first of these is the use of preincubation of peripheral blood mononuclear cells (PBMC) to decrease nonspecific T suppressor cell activity. We have found (Keller et al., Submitted; Keller et al., Submitted) that the blastogenic activity of cells increases and the percentage of FC $\gamma$  bearing T cells decreases employing this procedure. Furthermore, it permits the use of autologous cocultures which eliminates potential allogenic reactions in the classic coculture technique.

Secondly we employed low dose (15 rad) total body irradiation in the monkeys. In mice, this procedure has been shown to specifically and transiently ablate T suppressor cell activity (Anderson and Warner, 1976). Although similar studies had not been performed in monkeys prior to these experiments, biochemical, hematologic and clinical monitoring of the monkeys following irradiation did not demonstrate any untoward sequella of the procedure.

### EXPERIMENTAL RESULTS

The percentage of T cells, B cells, and macrophage-like adherent cells in the peripheral blood of symptomatic and asymptomatic human and non-human primates is summarized in Table 1. When studied, the groups were not acutely ill and their mean white blood cell counts and lymphocyte/monocyte percentages, as assessed by Wright-Giemsa-stained blood smears, were not significantly different (data not shown). In addition, the relative percentages of T cells, B cells, and macrophages were the same when groups were compared. Furthermore, the percentage of FC $\gamma$  bearing T cells is similar when human and non-human primate cohorts are compared. It should be noted, however, that our studies measured only total E rosettes and we were not able to measure these populations in bronchial lavage fluid due to the number of cells required.

We next examined mitogenic reactivity of PBMC from asymptomatic and symptomatic patients and monkeys using preincubation and cimetidine, a type two histamine blocker (Boren et al., 1977; Osband et al., 1979) to assess possible T suppressor cell influences in the cultures. These data are summarized in Table 2 for phytohemagglutinin, a predominantly T cell mitogen. Asymptomatic patients and monkeys reveal decreased peripheral blood mononuclear cell (PBMC) PHA stimulation (34% and 31%, respectively) compared to symptomatic cohorts. In the presence of  $10^{-3}$  cimetidine, however, asymptomatic patients and monkeys demonstrate an increased PHA responsiveness (31% and 10%) compared to fresh PBMC. Preincubation also results in increased PHA blastogenesis (51% and 70%, respectively). On the contrary, symptomatic patients and monkeys demonstrate a decreased response in the presence of  $10^{-3}$  cimetidine (-17% and -30%) or after preincubation (-16% and -12%) compared to fresh autologous PBMC. Similar differences were found when pokeweed mitogen, a predominantly B cell mitogen, was used (data not shown). As these results suggested possible differences in immunoregulation between symptomatics and asymptomatics, we examined the effects of similar procedures on antigenic reactivity. These data are summarized in Table 3.



TABLE 1. PERCENTAGES OF VARIOUS PBMC TYPES IN THE PERIPHERAL BLOOD OF SUBJECTS WITH HUMAN AND NON-HUMAN PRIMATE HYPERSENSITIVITY PNEUMONITIS\*

	<u>T</u>	<u>B</u>	<u>MONOCYTE</u>	<u>FC<math>\gamma</math>T</u>
HUMAN				
Asymptomatic	72 $\pm$ 4	11 $\pm$ 3	15 $\pm$ 4	19 $\pm$ 3
Symptomatic	73 $\pm$ 14	11 $\pm$ 3	17 $\pm$ 5	18 $\pm$ 3
MONKEY				
Asymptomatic	36 $\pm$ 3	11 $\pm$ 3	14 $\pm$ 3	8 $\pm$ 3
Symptomatic	19 $\pm$ 4	13 $\pm$ 3	16 $\pm$ 4	9 $\pm$ 2

\*T cells represent the number of PBMC per 100 mononuclear cells which bind 3 or more amino iso-ethyl-thiouonium bromide treated Sheep RBCs. B cells represent the number of PBMC per 100 cells that bind 3 or more immunobeads which have been covalently linked to polyvalent antihuman immunoglobulin. Monocytes are those cells per 100 PBMC that ingest the polyvalent antihuman Ig beads but have none bound to their surface. FC $\gamma$ T cells are those cells per 100 isolated T cells that bind three or more Bovine RBCs which were sensitized with the IgG fraction of rabbit anti bovine RBC antisera.

TABLE 2. THE EFFECTS OF PREINCUBATION AND CIMETIDINE ON THE IN VITRO PBMC BLASTOGENIC RESPONSE TO PHYTOHEMAGGLUTININ IN HUMAN AND NON-HUMAN PRIMATE HYPERSENSITIVITY PNEUMONITIS\*

	<u>Fresh PBMC</u>	<u>PBMC + 10<sup>-3</sup>M Cimetidine</u>	<u>Preincubation</u>
HUMAN			
Asymptomatic	71312	74033	108,184
Symptomatic	108479	90479	93,144
MONKEY			
Asymptomatic	54325	65185	92,149
Symptomatic	78688	54544	69,360

\*Values represent the arithmetic mean of the geometric means of triplicate samples of <sup>3</sup>H thymidine incorporation for each group stimulated with a maximum mitogenic concentration of phytohemagglutinin. The standard errors have not been included as they were <10% of the mean in all cases. Human groups were eight asymptomatic and eight symptomatics. Two asymptomatic and symptomatic monkeys were studied. The use of cimetidine and preincubation is explained in the text.

TABLE 3. THE EFFECT OF PREINCUBATION ON THE IN VITRO BLASTOGENIC RESPONSE TO PIGEON SERUM IN HUMAN AND NON-HUMAN PRIMATE PIGEON BREEDERS HYPERSENSITIVITY PNEUMONITIS\*

	<u>Fresh PBMC</u>	<u>Preincubated PBMC</u>
HUMAN		
Asymptomatic	5994	13366
Symptomatic	42698	23816
MONKEY		
Asymptomatic	27210	62881
Symptomatic	49082	23723

\*Values represent the arithmetic mean of the geometric means of triplicate samples of  $^3\text{H}$  thymidine incorporation stimulated with a maximum mitogenic concentration of pigeon serum before (fresh) or after (preincubated) a period of 48 hours in media alone. SE is not depicted as it represents <10% of the mean in all experiments. The groups are as in Table 2.

Fresh PBMC from both asymptomatic patients and monkeys demonstrate significantly reduced blastogenesis to a maximum mitogenic concentration of pigeon serum compared to symptomatic controls ( $p < .001$  and  $< .01$ , respectively). After preincubation, however, these same PBMC from asymptomatic patients and monkeys demonstrate a marked increase in blastogenesis to pigeon serum (122% and 131%, respectively). Symptomatics, on the other hand, demonstrate significantly higher initial blastogenic activity to pigeon serum than do asymptomatics but this reactivity decreases after preincubation. Taken together, then, asymptomatics differed significantly from symptomatics in their blastogenic activity to both mitogens and a specific antigen stimulus. These data, therefore, led us to determine which immunoregulatory cell types were different between the cohorts. These data are summarized in Table 4.

TABLE 4. EFFECT OF ADDING VARIOUS SUBPOPULATIONS OF AUTOLOGOUS PBMC ON THE PIGEON SERUM INDUCED BLASTOGENESIS OF PREINCUBATED CELLS IN HUMAN AND NON-HUMAN PRIMATE HYPERSENSITIVITY PNEUMONITIS\*

	HUMAN		MONKEY	
	<u>Asymptomatic</u>	<u>Symptomatic</u>	<u>Asymptomatic</u>	<u>Symptomatic</u>
Preincubated alone	13776	23816	62881	23723
Preincubated & T	5789	26612	40873	29890
Preincubated & Adherent	9298	12074	52506	20400
Preincubated & Pigeon Serum Induced	4466	31713	18850	41515

\*Values represent the arithmetic mean of the geometric mean of triplicate samples of  $^3\text{H}$  thymidine incorporation for autologous cocultures stimulated with a maximum mitogenic concentration of pigeon serum. The standard errors were not reported as they represent <10% of the mean in all instances. All mixtures were done at a 1:1 ratio using preincubated PBMC and autologous populations as above.

In both asymptomatic patients and monkeys, the addition of fresh T cells decreased the response of autologous PBMC to pigeon serum (58% and 36%, respectively). In addition, if PBMC from asymptomatics were first incubated with pigeon serum for 48 hours, irradiated to prevent proliferation, and added to autologous preincubated PBMC challenged with pigeon serum there was a marked decrease in blastogenesis (68% and 70%, respectively). Symptomatic PBMC cocultures from humans and monkeys, however, behaved quite differently. The addition to T cells caused a slight enhancement of pigeon serum induced blastogenesis (11% and 25%, respectively) and the addition of pigeon serum preincubated cells (see above), which should induce specific suppressor cells, actually caused a significantly enhanced response ( $p \leq .01$ ). The addition of monocytes, on the other hand, exerted similar suppressive effects in both symptomatic and asymptomatic patients and monkeys. Similar experiments were also performed using autologous cocultures stimulated with PHA or PWM (data not shown). The results of all experiments demonstrated absence of functional inactivity of both spontaneous T and pigeon serum induced suppressor cells in symptomatic patients and monkeys compared to the asymptomatic cohorts while monocyte suppressor cells were active in all groups tested.

As a result of these findings, we turned our attention to the monkeys where direct biologic experiments could be performed. In order to ascertain if suppressor elements were related to disease activity or merely epiphenomena, we chose to temporarily ablate T suppressor cell activity in the asymptomatic animals by low dose total body irradiation. If T suppressor cells were impor-

tant then these monkeys should behave differently to an intratracheal challenge with pigeon serum after TBI. The clinical data on all monkeys before and after irradiation and challenge are summarized in Table 5.

TABLE 5. PHYSIOLOGIC PARAMETERS IN NON-HUMAN PRIMATE HYPERSENSITIVITY PNEUMONITIS\*

<u>Procedure</u>	<u>Animals</u>	<u>Number of Animals Studied †</u>	<u>Temp °F</u>	<u>Resp/Min</u>	<u>WBC x 10<sup>3</sup></u>
Control	Asymptomatic	2	0.5	2.7	11.6
	Symptomatic	2	3.1	50	17.1
6 Days Post-Irradiation	Asymptomatic	2	2.5	16	27.5
	Symptomatic	2	3.0	36	27.2

\*Values represent the mean of the maximum change from baseline in temperature, respiratory rate, and WBC count. Each was measured before challenge (baseline) and at the period of maximum clinical response to bronchial challenge.

†Each symptomatic control animal was studied on 3 separate occasions, while the symptomatic control animals were studied on 2 separate occasions. Post-irradiation animals were studied once.

In three separate challenges of the two asymptomatic monkeys before irradiation, there was a negligible rise in both temperature and respiratory rate. The white blood cell count, however, increased significantly. Challenge of the symptomatic pair, on the other hand, resulted in a mean temperature rise of 3.1°F, marked tachypnea, and a polymorphonuclear leukocytosis. The differences for both temperature and respiratory rate are significant ( $p \leq .001$ ). Following 15 rads of total body irradiation, however, the previously asymptomatic animals became clinically ill after challenge, and all four monkeys developed similar significant increase in temperature, respiratory rate, and white blood cell count (see Table 5). In addition, one asymptomatic monkey that was challenged after total body irradiation developed rales on physical examination as did the symptomatic pair.

The irradiated monkeys were then allowed two weeks to recuperate and again challenged with pigeon serum. The results of these experiments are again shown in Table 5. At this time, the previously asymptomatic pair failed to develop physiological signs of clinical disease although leukocytosis was still noted. The symptomatic pair, however, still manifested signs of clinical disease. These data demonstrate that total body irradiation followed by bronchial challenge is associated with transient clinical disease in the asymptomatic pair.

Finally, we repeated the in vitro coculture studies to assess the effects of TBI on suppressor cell activity in monkeys. These data are summarized in Table 6. The in vitro coculture experiments also revealed similarity between previous asymptomatic and symptomatic groups after TBI. In fact, suppression of the response to pigeon serum by fresh T cells, pigeon serum, and Con A activated suppressor cells noted in in vitro asymptomatic cocultures before irradiation were abrogated by TBI. These results mimic the results in pre- and postirradiation cocultures of symptomatic PBMC. However, irradiation did not alter the ability of monocytes to suppress the response to pigeon serum in both asymptomatic and symptomatic cocultures.

TABLE 6. THE EFFECT OF ADDING VARIOUS SUBPOPULATIONS AUTOLOGOUS PBMC ON THE PIGEON SERUM INDUCED BLASTOGENESIS OF PREINCUBATED CELLS IN NON-HUMAN PRIMATE HYPERSENSITIVITY PNEUMONITIS AFTER IN VIVO TOTAL BODY IRRADIATION\*

	<u>ASYMPTOMATIC</u>	<u>SYMPTOMATIC</u>
Preincubated	50085	31947
Preincubated & T	67461	64425
Preincubated & Adherent	45848	30882
Preincubated & P.S. Induced	70678	69719
Preincubated & Con A Induced	76988	60379

*\*Values represent the arithmetic mean of the geometric mean of triplicate samples of  $^3\text{H}$  thymidine incorporation for autologous cocultures stimulated with a maximum mitogenic concentration of pigeon serum. The standard errors were not reported as they represent <10% of the mean in all instances. All mixtures were done at a 1:1 ratio using preincubated PBMC and autologous populations as above.*

These changes were corroborated by examining the effect of irradiation and intratracheal challenge on plasminogen activator (PA), a nonspecific indicator of pulmonary inflammation (Hamilton et al., 1976; Gordon et al., 1974) obtained from bronchoalveolar fluid. Before irradiation, asymptomatic animals demonstrated decreased plasminogen activator levels in response to an in vivo challenge with pigeon serum compared to prechallenge levels. After irradiation, however, asymptomatic animals responded as did symptomatics both before and after irradiation by demonstrating increased plasminogen activator levels after in vivo challenge. However, when the animals were allowed two weeks to recuperate, asymptomatic animals reverted to preirradiation status in that: 1) they developed neither fever nor signs of pulmonic congestion after challenge; 2) T cell pigeon serum and Con A activated T suppressor cells were again demonstrable in pigeon serum and mitogen activated cocultures, and 3) they demonstrated decreased PA activity in response to

an in vivo antigen challenge. Symptomatic animals, on the other hand, demonstrated no change in the clinical, in vitro coculture or PA activity after this period of recuperation compared to pre- or postirradiation challenge data.

## DISCUSSION

These data raise a number of important points. First they suggest that the dichotomy between antigen specific and nonspecific suppressor cells reported in murine studies (Tada et al., 1975) may not be relevant in non-human primate and perhaps human disease. In this regard, Rocklin has recently shown (Rocklin et al., 1980) that antigen specific suppressor cells in atopic patients bear histamine receptors which is the same population that is activated by Concanavalin A and inhibited by cimetidine (Milson et al., In Press; Osband et al., 1979). Our studies, as well, suggest that the nonspecific (Con A activated) and specific (pigeon serum-induced) T suppressor cells either act in concert or represent overlapping populations since they each suppress the response of cocultured PBMC to pigeon serum. Although these studies have not addressed this question directly, our findings suggest that further work on the relationship of nonspecific and specific suppressor T cells in non-human primate and perhaps human disease is warranted.

In addition, these studies raise a significant question about the general relevance of compartmentalization of the immune response within the lungs reported in sarcoidosis (Hunninghake and Crystal, 1981; Hunninghake et al., 1979). Our data show a strong correlation between clinical, physiologic, and biochemical evidence of pulmonary inflammation and the presence or absence of functional specific and nonspecific T cells in the peripheral blood. If it can be shown that peripheral blood immunoregulatory elements are involved in pulmonary pathology, studies of the relationship of immunoregulation to a variety of pulmonary diseases will be facilitated. At the present time, however, a definitive answer is not possible since the number of cells obtained by bronchial lavage was insufficient to perform the necessary parallel studies.

Our data further suggest a close relationship between human hypersensitivity pneumonitis and the monkey model. Previous studies have shown correlations between the clinical course, histologic changes, circulating antibody levels, and tests of cellular immunity between asymptomatic and symptomatic humans and monkeys with HP. The data presented here reveal close parallels between immunoregulatory cells function and clinical disease in the monkey model. In human pigeon breeders disease, the presence of spontaneous and pigeon serum-induced T suppressor cells correlates with a lack of clinical symptoms. There is no correlation, however, between clinical disease and the presence or absence of Con A activated T suppressor cell or FC $\gamma$  bearing T cells. In monkeys, the presence of spontaneous, Con A-induced, and pigeon serum-induced T suppressor cells correlates with the asymptomatic state while the FC $\gamma$  bearing T cell pool and adherent suppressors are present in both symptomatic and asymptomatic animals. The only difference noted, therefore, is the absence of Con A-induced suppressor cells in symptomatic monkeys and their presence in symptomatic patients. These correlations, although not total, suggest that the monkey model can be used to critically examine the immunoregulatory mechanisms involved in the pathogenesis of human HP.

Finally, the studies presented here suggest that T cell immunoregulation is an important factor in the pathogenesis of disease in the monkey model. Although the effects of low dose total body irradiation on the monkey have not been reported previously, studies in other species (Anderson and Warner, 1976) suggest that the abrogation of T suppressor cell function occurs at doses that have few other reported physiologic effects. In the studies reported here, there were no significant effects of 15 rads of TBI on the clinical behavior, serum chemistries, white blood cell counts, or adherent suppressor cell function in these monkeys. Furthermore, there was no evidence of any clinical manifestation or radiation sickness. Nonetheless, this dose of irradiation resulted in clinical hypersensitivity pneumonitis in both previously asymptomatic animals. This was confirmed by clinical, physiologic, and biochemical data and was associated with the disappearance of T suppressor cells from the peripheral blood of these animals. In addition, this effect was transient. Fourteen days after TBI, asymptomatic animals no longer responded to a bronchial challenge of pigeon serum with a fever, tachypnea, increased plasminogen activator release, or clinical signs of disease, and peripheral blood suppressor T cells were again functional in vitro (data not shown).

These studies, therefore, represent the first demonstration that an experimentally-induced inhibition of suppressor T cell function is associated with the development of clinical disease in primates. Furthermore, it confirms and strengthens the hypothesis that immunoregulation may be an important element in the pathogenesis of hypersensitivity pneumonitis. Nonetheless, these studies must be viewed with caution for a number of reasons. First, the disappearance of T suppressor cell activity in vitro associated with the appearance of clinical disease does not constitute proof that it was this effect of TBI which caused disease. Although this has been demonstrated convincingly in mice (Anderson and Warner, 1976), further evidence in primates is necessary, and studies are ongoing. Second, abrogation of T suppressor cell function, as well as the development of clinical disease, is quite transient. In murine studies, T suppressor elements do not reappear for one month after low dose TBI yet our data indicate that they are functional within two weeks. Whether this represents species differences of T suppressor elements, continued activity of monocyte suppressors after TBI, or a suboptimal dose of TBI cannot be answered by the present study. Finally, the correlation between suppressor cell elements in the monkey and human hypersensitivity pneumonitis is not absolute. Further studies will be necessary to conclusively establish the relevance of these findings to the pathogenesis of human disease. Despite these reservations, these data do provide evidence that the monkey is a valid and important model of human HP and that immunoregulation is an important, albeit incompletely delineated element of the pathogenesis in this spectrum of disease.

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MULTIPLE BIOLOGICAL MARKERS IN THE  
STUDY OF CONSTITUTIONAL SUSCEPTIBILITY

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Biochemistry, immunology, clinical medicine, and host factors have all been utilized in the study of risk factors to assess constitutional susceptibility to environmental agents (Tomatis, 1980; Saffiotti, 1980; Wigle et al., 1980). In the past few years, we have studied multiple biological markers in human populations in an attempt to evaluate their value as determinants of cancer risk (Guirgis et al., 1978; Guirgis et al., 1979).

A wide variety of biological markers have been previously studied by several investigators. The markers include: a) immunochemical parameters such as carcinoembryonic antigen (CEA) which has been associated with cancer of the colon and other malignant neoplasms (Cooper et al., 1979; Gold and Freedman, 1965); alpha fetoprotein (AFP) which has been found to be associated with hepatomas (Abelev, 1971); serum immunoglobulins (IgG, IgM and IgA) that were found to be abnormal in patients with breast cancer and other tumors (Roberts et al., 1975); human chorionic gonadotropin (HCG) in seminomas (Gips et al., 1980) and others; b) biochemical factors such as arylhydrocarbon hydroxylase (AHH), glucose-6-phosphate dehydrogenase, LDH and others (Guirgis et al., 1977; International Agency for Research on Cancer Monograph, 1977); c) measurements of immunocompetence in vivo, e.g. skin testing with common and non-specific allergens, e.g. DNCB (Giuliano et al., 1979); d) phenotypic physical signs such as rectal polyps and osteomas in Gardner's Syndrome where there is an increased risk for colon cancer (Gardner and Richards, 1953).

When one looks closely at the value of individual biological markers in the diagnosis of malignant neoplasms, it becomes clear that none of them possesses the level of specificity and sensitivity to serve that purpose. For example, carcinoembryonic antigen which was thought to be specific for identification of subjects with colon cancer, was also found to be elevated in other patients with other malignant neoplasms. CEA, however, has been a useful marker for prognosis of cancer of the colon as well as other malignancies.

Arylhydrocarbon hydroxylase (AHH) was studied by several investigators in relation to lung cancer. Several reports showed that lung cancer patients had a high AHH inducibility when compared with controls (Kellerman et al., 1973; Emery et al., 1978).

In our own studies, we have looked at several markers singly and collectively in cancer patients and in subjects at high cancer risk. The markers studied include: total lymphocytes, T and B lymphocytes; immunoglobulins, IgA, IgG and IgM; AHH; and CEA. Using different tumor models, we found that it was inefficient to utilize each of the markers individually. We have developed a statistical model in order to combine those markers in the derivation of an index (Table 1). As can be seen from Table 1, the probability that an observed value is more extreme than the standard normal population mean is calculated for each marker. Then probabilities of all the markers for each individual are combined to derive a risk index. Assessment of the significance of a high index is performed by comparison to the 95th percentile of a chi square distribution.

TABLE 1. METHOD OF THE DERIVATION OF INDEX

- |  |  |
|--|--|
| A. OBSERVED VALUE OF EACH BIOMARKER  | $x_{ij}$   |
| B. OBTAIN A Z SCORE USING A STANDARD POPULATION DISTRIBUTION<br>$i = 1, 2, \dots$ NUMBER OF BIOMARKERS<br>$j = 1, 2, \dots$ NUMBER OF SUBJECTS | $z_{ij} = (x_{ij} - \mu_i) / \sigma_i$   |
| C. CALCULATE THE PROBABILITY OF OBTAINING A MORE EXTREME STANDARDIZED NORMAL VALUE FOR EACH $z_{ij}$   | $p_{ij}$   |
| D. COMBINE PROBABILITIES FROM TESTS OF SIGNIFICANCE (FISHER 1970) FOR EACH SUBJECT<br>$K =$ NUMBER OF VARIABLES INCORPORATED IN THE INDEX      | $p_{1j} p_{2j} \dots p_{kj}$   |
| E. TRANSFORMATION OF THE PRODUCT BY TAKING LOGARITHM OF AND MULTIPLY BY $-2$   | $I_j = -2 \ln \prod_{i=1}^k p_{ij}$ <p>OR</p> $I_j = -2 \sum_{i=1}^k \ln p_{ij}$ |
| F. ASSESS FOR SIGNIFICANCE BY COMPARISON TO 95TH PERCENTILE OF $\chi^2$ DISTRIBUTION WITH $2K$ DEGREES OF FREEDOM                              |  |

The criteria to develop an index of biological markers are: 1) the choice of variables which would make biological sense; 2) the final set of variables should be composed of markers which are not severely correlated to prevent confounding effects; 3) each of the markers should be tested for its level of

contribution into the index. Some of the markers did not add to the index. Such markers were eliminated from consideration in the derivation of the final index. 4) The separation between subjects with a significant index should be achieved in order to consider the use of multiple biological markers in assessment of risk for a particular tumor.

Using the above method, we have studied 3 models; first is a kindred where members are at high risk for cancers of the colon and of the endometrium (Lynch et al., 1977). This syndrome has been identified by Lynch (1976) as the Cancer Family Syndrome. The likelihood of a genetic mode of transmission in this family was found to be consistent with an autosomal dominant mode of inheritance (Figure 1). Using a life table one can calculate the probability of unaffected individuals who are offspring of affected parents and who are expected to develop cancer of the colon or endometrium (taking into consideration their age at the time of testing, sex, and their position within the kindred). Individuals at risk in the pedigree in Figure 1 were studied for the above-mentioned biological markers, (CEA, T cell, B cell, PHA-mitogen activation, AHH activity and serum IgA, IgM, and IgG) as well as smoking status. The index was calculated for each individual who was tested. There were 4 individuals who exhibited a high index score from the 19 individuals examined. As can be seen from Figure 2, the bar graph shows that those four who had a significantly higher score had a mean which was different from others studied with a probability of misclassification of 3%. A follow-up of this kindred is of course the only way that we can assess the predictive value of the index of multiple biological markers and the success of this approach to identify individuals at high cancer risk. At the time we examined this family, a life table analysis indicated the expected number of family members to develop cancer was 4.7.

The second model used to assess the value of the multiple biomarker index was a geographic cluster of malignant melanoma where we tested 8 biological markers, many of which were the same as in the previous model. We examined 15 malignant melanoma patients, their spouses and their children. All the blood samples were collected on the same day to insure that there was no variability due to laboratory error. The markers used to provide an index of susceptibility were T lymphocytes, PHA-mitogen activation, IgM and IgG. Five melanoma cases and three spouses had significant scores (Figures 3 and 4). The fact that 3 of the spouses of malignant melanoma cases have shown a significant index score might indicate a connubial effect. This observation was confirmed by two other investigations (Dean et al., 1979; Vandebark et al., 1979). In our study we also found that serum cytotoxic antibody to melanoma cell lines was detected in 42% of the melanoma cases and 63% of the spouses (Guirgis et al., 1979).

The third model we studied in relation to the biomarkers was in monozygous and dizygous twins. The purpose was to quantitate the variability of the phenotypic expression of those markers and to examine genetic versus environmental influence on their levels. For this purpose, we have studied 17 monozygotic twins and 5 dizygotic twins. The laboratory markers studied include T and B cells and subpopulations of T cells; suppressor T cells ( $T_{\gamma}$ ) and helper T cells ( $T_{\mu}$ ), CEA and the other fetal antigens (AFP and HCG), immunoglobulins

IV

III

II

I

II

III

IV

## LEGEND

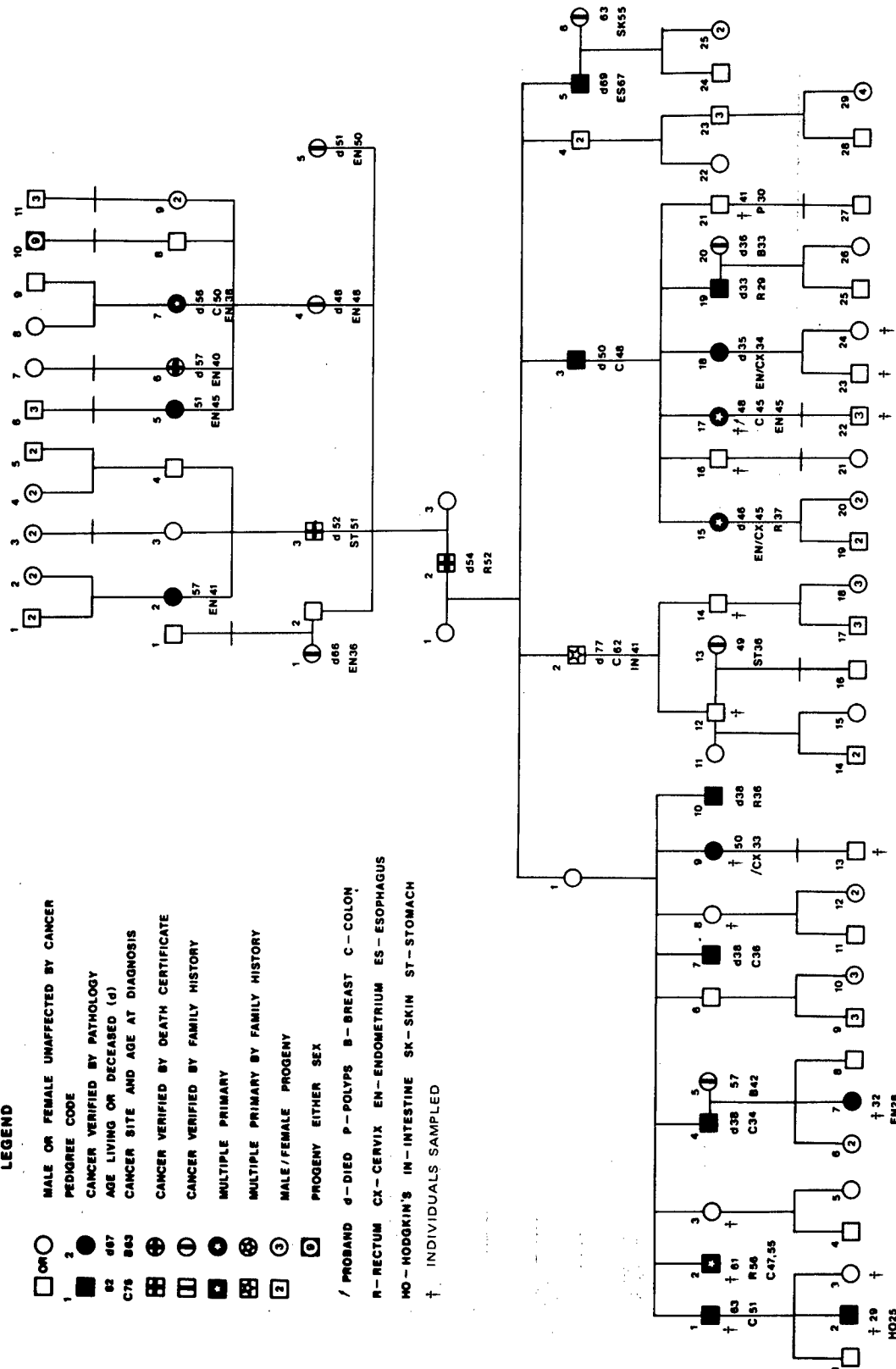
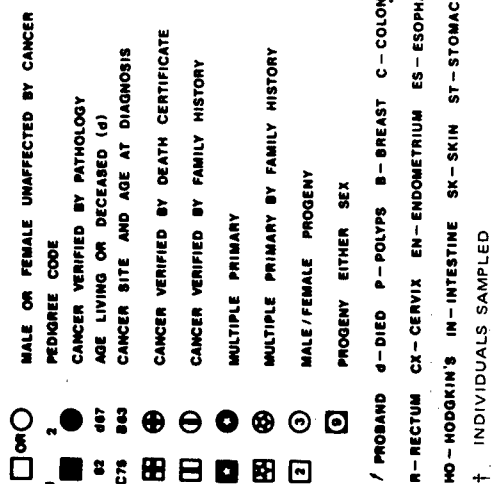


Figure 1.

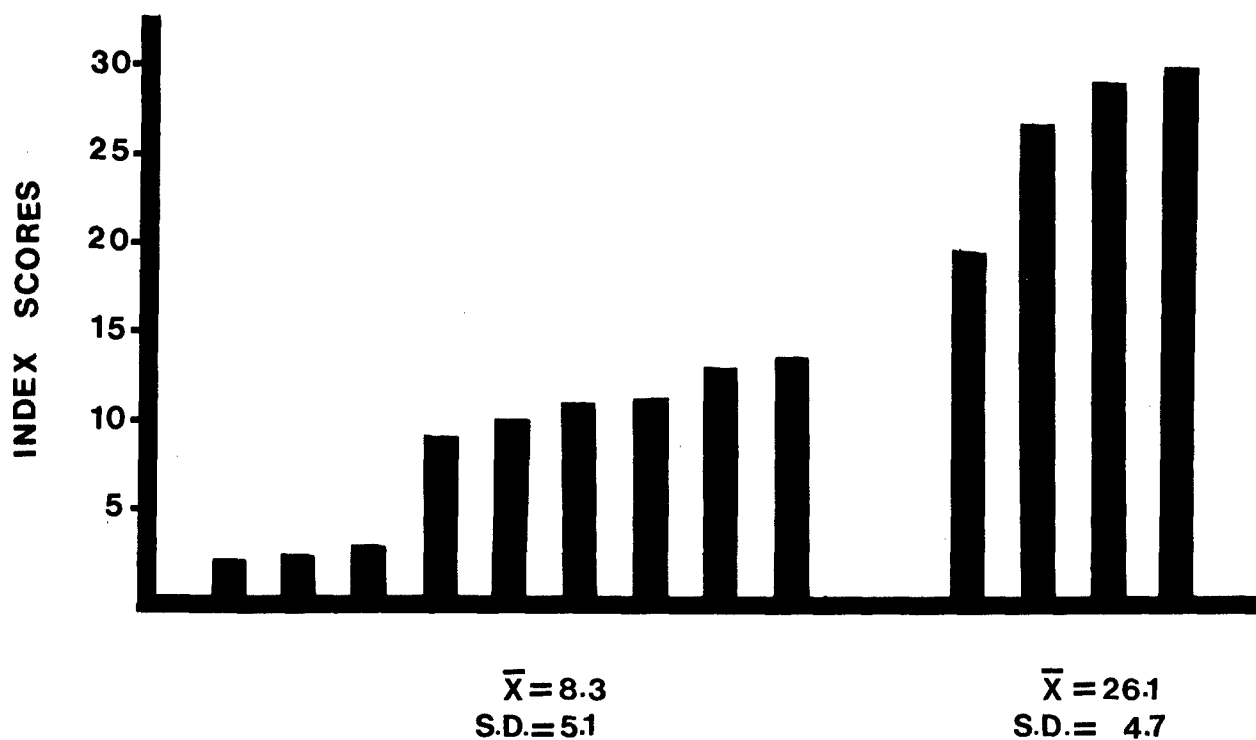


Figure 2. Index scores in progeny of affected parents.

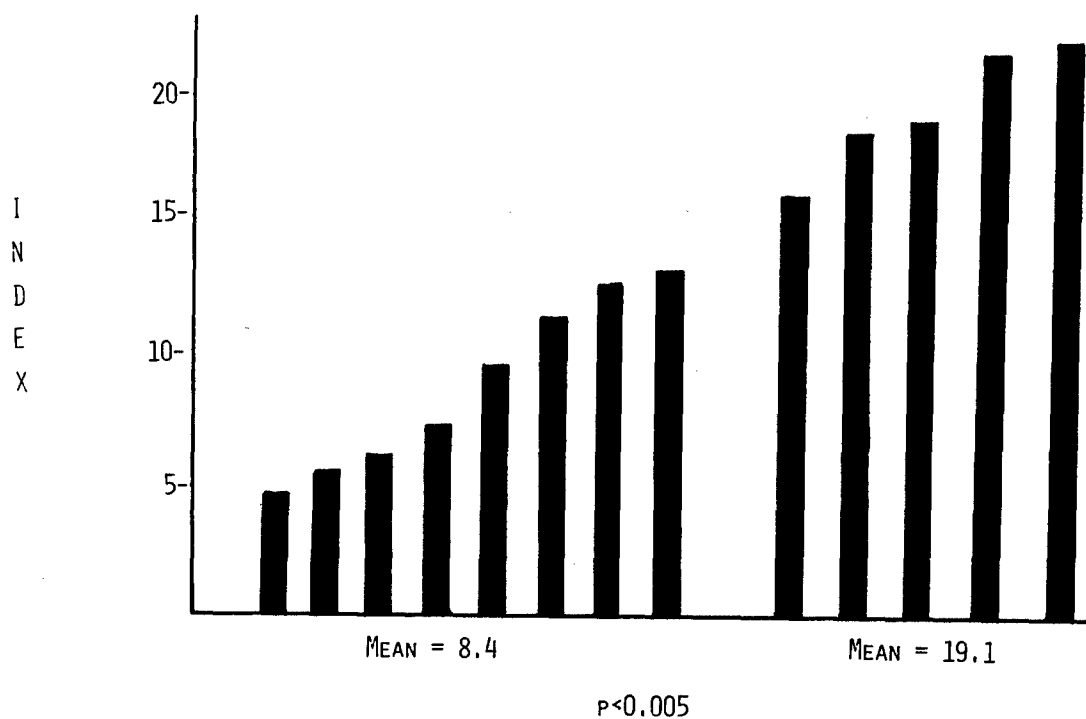


Figure 3. Index scores in malignant melanoma cases.

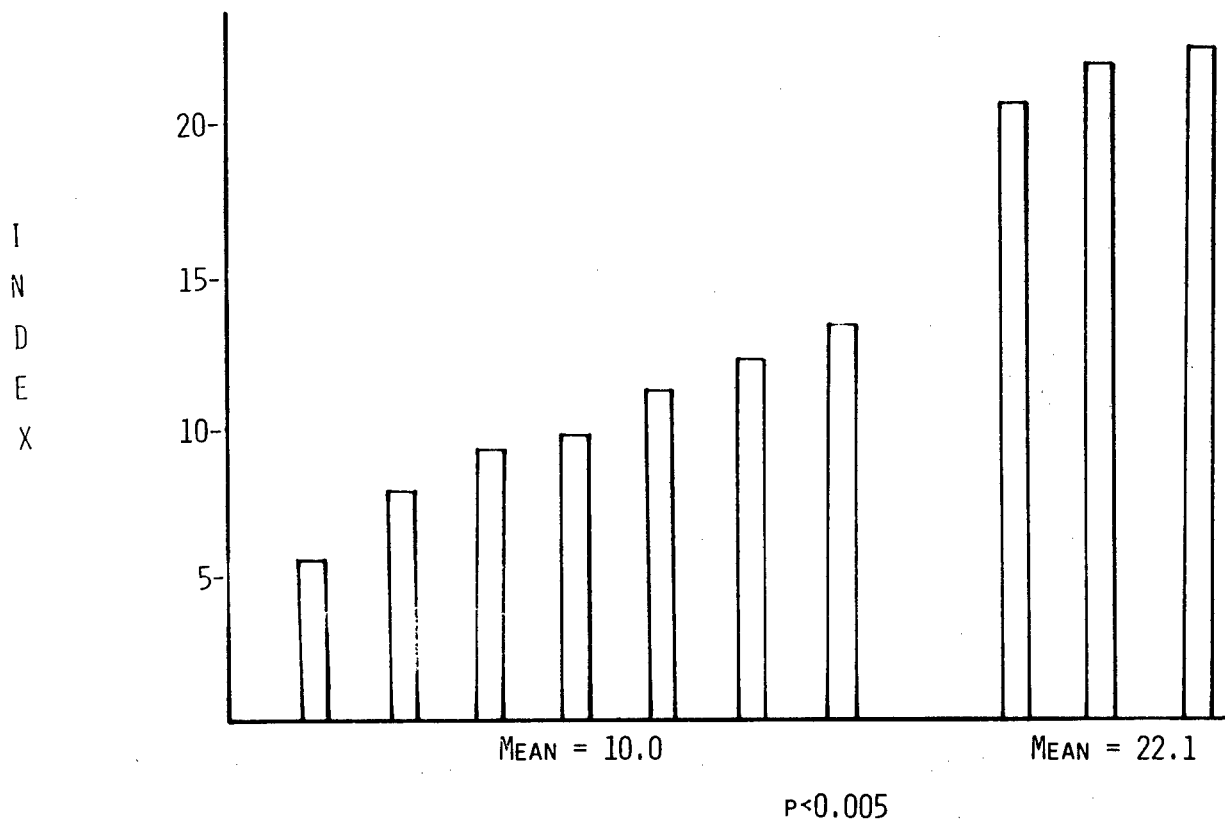


Figure 4. Index scores in spouses of malignant melanoma cases.

(IgG, IgA and IgM), PHA-mitogen activation and sister chromatid exchange. There was no significant age effect on the levels of the markers except in case of mitogen activation where we found that there is an effect of age and sex. Females exhibited a higher mitogen activation level than males. We also found that the frequency of T cells was somewhat dependent on age and sex and that females had higher T cell number than males. The temporal variability, over a six month period, showed that none of the markers have a mean difference which was above one standard deviation. Differences between monozygotic and dizygotic twins for each of the markers showed that dizygotic twins had consistently higher mean difference than monozygotic twins. In other words, more variability was observed in dizygotic twins. Therefore we attempted to study a heritability index for each of the markers studied. The data on heritability are shown in Table 2. The heritability index was significantly high in T cells, IgM and mitogen activation. It seems that there is a genetic component in the phenotypic expression of those three markers more than in any other.



TABLE 2. HERITABILITY INDEX (H)

VARIABLE	MZ		DZ		H	
	$(\bar{X}^2)_d$	$V_{d_1}$	$(\bar{X}^2)_d$		$V_{d_2}$	$\frac{(V_{d_2}-V_{d_1})}{V_{d_2}}$
T Lymphocytes	86.2 ± 114.3	43.1	413.8 ± 457.1		206.9	0.792*
T $\gamma$	44.5 ± 59.9	22.3	48.5 ± 38.2		24.3	0.083
T $\delta$	185.9 ± 248.1	92.9	229.8 ± 171.2		114.9	0.191
IgG	26220 ± 39,236	13110	37,440 ± 39,196		18720	0.2997
IgA	2484 ± 2971	1242	3511 ± 5877		1756	0.292
IgM	509 ± 1039	255	5119 ± 8224		2559	0.901*
Mitogen Activation (MA)	427128 ± 655,161	213,564	1,919,998 ± 1,746,277		959,999	0.778*

\*Indicates a significant genetic component contributing to the phenotypic expression of these markers.

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## OPEN FORUM

MAJ. ALLEN (Air Force Environmental Health Laboratory): I have a question for Dr. Kouri in regard to the Houston study. In measuring the AHH activity, did you collect any prefreeze control data to see how much activity was lost due to freezing in liquid nitrogen? Could protein denaturation due to thawing and freezing mask some significant increase or decrease in the enzyme activity?

DR. KOURI (Microbiological Associates, Incorporated): In the specific experiments in the Houston study, we didn't do any fresh samples. In about a hundred and fifty other individuals in which we did simultaneous fresh versus frozen samples, we got a very significant correlation between the AHH level found in the fresh and that found in the frozen sample. There was no diminution in AHH activity for multiple samples from the same individual kept frozen for about a year. I don't think that was a problem, although they weren't done specifically with that study. Lymphocytes from lung cancer patients could perhaps be more fragile or of different propensities in terms of stability than those of normal individuals; that still is a possibility.

DR. DUNGWORTH (University of California, Davis): Dr. Keller, what was the level of precipitating antibody in the two symptomatic versus the two asymptomatic monkeys?

DR. KELLER (Milwaukee Children's Hospital): It was equivalent using the dilution technique. The same has been found in terms of human patients as well. Although there are minor differences, if one looks at a cohort there really are no differences between the levels of precipitating antibodies.

DR. NEWTON (University of California, Irvine): Dr. Cohen, you showed data that indicated there was a higher incidence of pulmonary dysfunction in the family members which you could not explain. You adjusted for smoking but did you also look at whether the family member that had the dysfunction was also a smoker?

DR. COHEN (Johns Hopkins School of Hygiene and Public Health): You're talking about the problem of passive smoking?

DR. NEWTON: Yes.

DR. COHEN: There is really no way that we can evaluate this factor. We looked at whether the individuals we were studying for pulmonary function were smokers or had never smoked. However, passive exposure to smoking in the home and also in the workplace is very difficult to evaluate. There are some very interesting studies about passive smoking and the effects on children's pulmonary function. Exposure to passive smoking is one thing that is extremely difficult to evaluate in an adult. An individual may have had parents who had never smoked but still have grown up and married a spouse who smokes or be exposed to smoke in the workplace, travel or other public places. It is an interesting problem that we haven't solved. If you have any suggestions as to how that could be resolved, I'd really appreciate them very much.

DR. BERCZ (U. S. Environmental Protection Agency): Dr. Keller, what was the role of IgE mediated hypersensitivity in your cases? Did you try to do such studies? What kind of monkeys did you work with?

DR. KELLER (Milwaukee Children's Hospital): There is no evidence to suggest in these hypersensitivity pneumonidities that IgE is a factor. In fact, the preponderant immunoglobulin is IgG. There are also lesser levels of IgA and IgM. The monkey used was a stump-tail monkey which is probably not the best model. We now are planning to use a Rhesus model because the immunologic parameters of the Rhesus and man are a lot more comparable than they are between the adult stump-tail and man.

DR. BERCZ: Well, the question still resides that there are a number of cases of IgE mediated in your response situation in which the total immunoglobulin is not elevated.

DR. KELLER: Absolutely correct.

MR. VERNOT (University of California, Irvine): Dr. Cohen, if I understood your hypothesis, there was a possibility many of the non-pulmonary pathological elements associated with cigarette smoking were precipitated by the lung lesion. If that's true, the effect of cigarette smoking on cardiovascular problems disappears or is moderated significantly shortly after cessation of smoking whereas the lung effects are rather longstanding. Does that affect your hypothesis?

DR. COHEN (Johns Hopkins School of Hygiene and Public Health): Pulmonary dysfunction would not necessarily be a precipitating factor but rather a cofactor. It may be a precipitating factor in some cases. As far as the effects of cessation of smoking, in most of the studies with smoking cessation there is an improvement in pulmonary function or at least an absence of further deterioration. Except for mortality in the first year after cessation of smoking, mortality from most causes tends to level off.

DR. MASTROMATTEO (Inco Limited): Dr. Guirgis, you said you were trying this method of biological markers with shipyard workers. In those workers who have a high index, how could you differentiate whether they may be in risk of developing mesothelioma or carcinoma and of which site? What would you do to follow up these workers who have a high index?

DR. GUIRGIS (University of California, Irvine): What we're doing right now is to epidemiologically assess the predicted number of shipyard workers who are going to develop mesothelioma, lung cancer and GI tract cancers using a computer model. The first step is to see if we can actually come up with a close estimate of the number of individuals who are going to exhibit a significant index of some sort. Those are the people that we should follow up at a later time. I think it needs a lot of patience and a lot of persistence, but that's the only way I see in order to assess the usefulness of biological markers. All we are doing right now is to identify those workers who are susceptible.

DR. MACH (The Aerospace Corporation): Dr. Keller, can immunological differences or changes in immunological response be used as a probe of irradiation? What about effects of inhalation of uranium dust on specific organs, for example?

DR. KELLER (Milwaukee Children's Hospital): I don't know whether it really would be possible. I suppose it could if you had the control population to work with but you have to have all the data beforehand if you are going to use a change in immunologic response as a probe. That is, a prospective study. The response to irradiation varies significantly in a dose dependent fashion. In a non-experimental or uncontrolled situation, the effects of irradiation tend to be so broad that it would be very difficult to pick out exactly what was going on. I don't think that immunologic responsiveness would be a good probe for radiation exposure unless the radiation exposure is at very low levels.

MR. JONMAIRE (Uniroyal, Incorporated): Dr. Kouri, given the variation in AHH activity that you described in animals, what's the most correct microsomal fraction to use for activating chemicals in in vitro mutagenicity testing?

DR. KOURI (Microbiological Associates, Incorporated): There's really not an answer for that. In most of the genetic toxicology short-term model systems, about 50% of the known chemical carcinogens require some sort of metabolic activation of which this enzyme system we're talking about is probably one of the best. Ames originally described an Arochlor 1254 induced rat liver S9 fraction as an activation system. As far as we're concerned, the S9 fraction has been as good as anything else we've used. In certain instances with certain chemicals, rat liver S9 gives the worst result and in other conditions the best although there is not really a very good data base yet to support that statement. If there's any black box in genetic toxicology assays it really resides within the question of which type of enzyme system should be used. This enzyme system is really a detoxification enzyme system first and an activating system second. Under most conditions you metabolize compounds fairly efficiently to water soluble forms so they can be excreted from the body. Under fairly unique conditions it has been turned into an activating system. We've maximized or optimized that response and find a variety of compounds that we reported here. If you get the right combination of activating and inactivating enzyme systems you'll metabolize methylcholanthrene very quickly to water soluble inactive forms and you'll not get any activation. I went around your question to say that there really isn't a good answer.

DR. LaDU (University of Michigan Medical School): Dr. Kouri, since this test on the benzopyrene hydroxylase is such a common one and so controversial at the moment, what are your thoughts in regard to the difference between levels of benzopyrene hydroxylase activity and inducibility of benzopyrene hydroxylase activity? Early studies of Kellerman, Kellerman and Shaw and subsequent studies seem to emphasize inducibility as a genetic trait and the highest degree of inducibility is when your numerator is large but your denominator is small. In terms of the data you were showing, what was the level of enzyme after induction? There are really two things you're looking at: inducibility and level of benzopyrene hydroxylase before and after induction. I wonder how you feel about those two different properties. I don't know any

reason why inducibility should be a genetic marker, but that should be as important perhaps as the enzyme activity itself.

DR. KOURI (Microbiological Associates, Incorporated): The original studies that you just quoted suggested that inducibility provided, in fact, a little bit better reproducibility - better reproducibility in terms of inter-assay or inter-individual variation. It's true that the activity you observed in mitogen activated human lymphocytes is very much a reflection of how you grew the lymphocytes. We could get any sort of inducibility that we wanted depending on how we grew the cells. These data were produced from cells grown in human A/B serum, not fetal calf serum that had seemingly very low levels of these endogenous inducers. Our inducibility ratios were normally on the order of between 8 and maybe 20 fold. That's what these would also be. Dr. Kellerman and other workers selected a fetal calf serum lot which gave them a certain fairly high level of non-induced or constituent enzyme activity and then looked above and beyond that as a marker. I don't argue with that as a marker technique or as a mechanism; however, it is very difficult to compare data in different laboratories based on that technique. Relative inducibility poses many problems in trying to compare interlaboratory variations and that's the reason we went right back and used specific activity.

DR. MEIER (Jackson Laboratories): Dr. Kouri, there is the possibility that inducibility is due to the regulatory gene versus levels that may be due to a structural gene.

DR. KOURI: That could be. Right now we really can't address that possibility because of the physiological problems associated with doing that assay itself. In the mouse that's in fact the case. There are situations in which inducibility very definitely in the mouse and the rat and in the rabbit is a reflection of the induced enzyme activity and not a diminution of the non-induced enzyme activity.

DR. COHEN (Johns Hopkins School of Hygiene and Public Health): Dr. Guirgis, do you ever see your multiple biological markers being used as screening tests for job applicants? When you screen shipyard workers and you find one that seems highly susceptible, do you tell them to look for work elsewhere because this is too dangerous a job situation?

DR. GUIRGIS (University of California, Irvine): Those are difficult questions to answer. In a shipyard I don't think there would be too much of a problem because there are positions where there isn't much exposure to asbestos. Furthermore, the levels of exposure now are very low and both management and labor are very much aware of the problems. I see it as a surveillance mechanism where you would look at the workers as a pre-employment test and then follow-up periodically because some of the biological markers are highly variable due to environmental factors. In some of them the variability is due to genetic effects. However, I'm not sure that we will be able to tell people that they are more susceptible than others for at least ten years.

DR. GUIRGIS (University of California, Irvine): Dr. Kouri, in your last slide on lung cancer, was there a correlation between the level of AHH and the tumor load or prognosis for survival or metastasis in those patients?

DR. KOURI (Microbiological Associates, Incorporated): There was no relationship between AHH activity and the type of tumor, the location of the tumor, the size of the tumor, or its eventual prognosis. We looked to see if there was any relationship of AHH activity in individuals who had a family history of lung cancer and found that they were not correlated. The only thing that correlated with the AHH activity was in individuals who had a family cancer history where the mean age at onset of lung cancer was about 8 years younger than the other lung cancer patients.